QP
1
J88
SEP 20 60
SCIENCE LIBRARY
PR

THE JOURNAL OF PHYSIOLOGY

vol. 153
no. 1
THE UNIVERSITY OF MICHIGAN
SEP 20 1960
SCIENCE LIBRARY

EDITED FOR

THE PHYSIOLOGICAL SOCIETY

BY

N. AMBACHE
R. V. COXON
M. DE B. DALY
P. M. DANIEL
S. P. DATTA
G. GORDON
G. W. HARRIS
R. C. JORDAN
B. KATZ
R. J. LINDEN
H. R. NOLTIE
W. J. O'CONNOR
W. D. M. PATON
W. A. H. RUSHTON
G. S. BRINDLEY
(*Secretary*)
R. D. KEYNES
(*Chairman*)

August 1960
Volume 153, No. 1

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Price 30s. net
(U.S.A. $5.00)

PAGE

Barer, Gwenda, R. Peritoneal drainage into the lymphatics of the diaphragm demonstrated *in vivo* by radiography 1*P*

Bannister, W. J. and Kay, R. H. A transistor stimulator for class practical work. 1*P*

Harvey, R. J., Kay, R. H. and Matthews, P. B. C. Recording the discharge of a single muscle spindle afferent by means of a dekatron scaler 3*P*

Gordon, G. and Kay, R. H. A thermo-electric 'cold' stimulator 3*P*

Agarwal, K. C. and Rodger, F. C. The effect on corneal nerve fibres of a reduction in tissue osmotic pressure 5*P*

Saxby, O. B., Siddiqi, S. and Walker, J. M. Continuous superfusion by means of a simple roller pump 6*P*

Smalley, B. A simple spirometer modification for recording volume changes electrically 7*P*

Axelsson, J. and Bülbring, Edith. The effect of removal of glucose from the medium on the electrical excitability of smooth muscle 8*P*

Barker, D. and Ip, M. C. The primary and secondary endings of the mammalian muscle spindle 8*P*

Andrews, W. H. H. and Trent, Margaret. The estimation of sulphobromophthalein (BSP) in turbid and grossly haemolysed plasma and in liver 11*P*

Leach, E. H., Parsons, J. A. and Paton, W. D. M. Pharmacologically active substances in a caecal sac of the male Garden Tiger moth 12*P*

Ough, A. J. A simple method for restraining rabbits during pyrogen assays. . 12*P*

Phillips, C. G. and Schuster, E. H. J. An instrument for charting the brain surface 12*P*

Cosgrove, Peggy, J., Cozon, R. V. and Spencer, Ann. Aid to alignment of catheter for insertion into abdominal vessels 12*P*

Engel, W. K. Sites of cholinesterase activity in cultured chick-embryo skeletal muscle cells 12*P*

Gordon, G., Landgren, S. and Seed, W. A. Responses of single cells in the caudal part of the spinal nucleus of the trigeminal nerve of the cat 12*P*

Daly, I. de Burgh and Waaler, B. A. Bronchial circulation and pulmonary vascular resistance (PVR) in isolated perfused lung lobes 14*P*

Whittam, R. Glycolysis and potassium transport in kidney slices from new-born animals 15*P*

Dawes, G. S., Mott, Joan C. and Stafford, Anne. Prolongation of survival in the anoxic foetal lamb 16*P*

Burn, J. H. and McDougal, D. Release of noradrenaline from artery walls as a cause of gangrene 17*P*

Blaschko, H. Primary and secondary amines as substrates of amine oxidases . 17*P*

Barclay, J. A., Hamley, E. J. and Houghton, Helga. Effect of pressure on heart and perfusion rates of the Langendorff preparation 18*P*

Ginsborg, B. L. and Mackay, B. The latissimus dorsi muscles of the chick . . 19*P*

Glees, P. and White, W. G. A study of skin absorption effects of tri-ortho-cresyl phosphate (TOCP) in hens 20*P*

Barber-Riley, G. The competitive effect of probenecid on bromsulphalein uptake in rat and dog liver slices 22*P*

Boyd, I. A. The diameter and distribution of the nuclear bag and nuclear chain muscle fibres in the muscle spindles of the cat 23*P*

Draper, M. H., Friebel, H. and Karzel, K. Calcium ions and the resting potential of frog sartorius fibres in 0·7 % NaCl solution 25*P*

Schild, H. O. Structure of acetylcholine receptors in intestinal smooth muscle . 26*P*

Zbrozyna, A. W. Defence reactions from the amygdala and the stria terminalis . 27*P*

ERRATUM

BALFOUR, W. E. & TUNNICLIFFE, H. E. (1960).
J. Physiol. **153**, 179–198.

P. 196, l. 3 of Summary: the insertion '(TBP)' was added as the result of an error in the editorial office and should be deleted.

J. Physiol. (1960), **153**, *pp.* 1–8
With 4 text-figures
Printed in Great Britain

475
8813A

ON THE EXISTENCE OF SPECIFIC SECRETORY SYMPATHETIC FIBRES FOR THE CAT'S SUBMAXILLARY GLAND

BY N. EMMELIN AND J. ENGSTRÖM
From the Institute of Physiology, University of Lund, Sweden

(*Received* 13 *January* 1960)

Stimulation of the cervical sympathetic trunk in cats causes a secretion from the submaxillary gland. The magnitude of the response varies in different cats. It may be almost as pronounced as that evoked by stimulation of the chorda tympani, but usually it is much smaller and in exceptional cats no secretion at all is obtained. Unlike the secretion elicited by stimulation of the chorda it is usually described as short-lasting, tending to cease in spite of continued stimulation.

Since the time of Ludwig, Claude Bernard, Heidenhain and Langley the sympathetic trunk has been assumed to carry specific secretory fibres for the gland. Kuntz & Richins (1946) found, however, in histological investigations that the innervation of the gland cells is mainly, perhaps exclusively, parasympathetic. In a later paper (Richins & Kuntz, 1953) the same authors expressed the view that the secretion following sympathetic stimulation is caused by the chemical transmitter released from the endings of the vasomotor fibres and diffusing to the gland cells. This conception was based on the observation that the secretory effect of sympathetic stimulation was abolished by occlusion of the external carotid artery. The theory seems to be an extension of the idea discussed by Cannon & Rosenblueth (1937), according to which some cells are excited by transmitter diffusing from nerve endings in contact with certain innervated 'key cells'.

The theory of Kuntz & Richins might afford an explanation of the facts that the sympathetic secretory effect appears with a fairly long latency, shows great variations in different cats, is obtained predominantly at the beginning of a stimulation period, and can be elicited by repetitive stimulation only. There is, however, no direct evidence to show that specific sympathetic secretory fibres do not exist.

Analogous problems have been discussed in connexion with other organs. Celander (1959) found in experiments on adrenalectomized cats no inhibition of the intestinal motility on stimulation of the splanchnic nerves, except when frequencies of stimulation were used which according to Folkow (1955) are above the physiological range. Similar observations

were made by Kock (1959). The conclusion was that the splanchnic nerves contain no specific inhibitory fibres for the intestine.

The experiments of the present paper arose from an investigation on supersensitivity of the gland cells following sympathetic denervation. It is known that removal of the superior cervical ganglion causes an increased responsiveness of the gland cells to chemical agents (Simeone & Maes, 1939). This fact seems to indicate that the sympathetic normally exerts some kind of action on the gland cells; this action could of course be indirect, via the vasomotor fibres, but it must then be due to an impulse rate within the physiological range.

From such considerations experiments on the effect on the gland cells of stimulation of the sympathetic trunk with impulses of low frequency seemed required; for in the earlier investigations on the secretory effect of sympathetic stimulation frequencies above what is now considered as physiological in the vasomotor fibres have been used. Such experiments are the subject of the first part of the present investigation. In the experiments of the second part the effect on the secretory cells of artificial stimulation of the cut sympathetic trunk has been studied by using a frequency of stimulation which restores the tone of the vessels of the gland existing before the nerve was cut. In the third part an intense vasoconstriction via the sympathetic trunk was elicited reflexly and the effect on the gland cells of this constriction and of artificial stimulation causing a similar constriction was investigated. The reflex activity was obtained by bleeding. A preliminary report of these experiments has been given (Emmelin & Engström, 1959).

METHODS

The experiments were carried out on cats anaesthetized with chloralose (about 80 mg/kg intravenously) or chloralose–urethane (60 + 100 mg/kg intravenously). At the beginning of the experiment the chorda tympani was cut. The submaxillary duct was cannulated and the drops secreted were recorded electromagnetically on the smoked drum by an assistant; in some experiments an ordinate recorder was used. The flow of blood from the gland was recorded as drops of blood from a cannula in the external jugular vein, after injection of heparin and ligation of tributaries not draining the gland. The drops passed a phototube counter, which operated an ordinate recorder. The blood was reinjected at intervals through a cannula in a femoral vein. For stimulation of the sympathetic trunk square-wave shocks of supramaximal strength and 1 msec duration were used.

In the experiment on the reflex response to bleeding blood was withdrawn from a cannula in a femoral artery. In order to prevent the bleeding from interfering mechanically with the flow of blood through the gland, and to prevent catechol amines released from the adrenals during the bleeding from reaching the gland, a separate perfusion of the gland from another cat was arranged. For that purpose an anaesthetized cat was heparinized and blood directed from the central end of a carotid artery through a polythene tube into the peripheral carotid of the recipient animal; in this cat all the branches of the carotid except that of the gland had been tied. The polythene tube was of such a size that it could be pushed through the

carotid up into the external maxillary artery to fit tightly into this vessel. A ligature round this artery and the tip of the tube was then often not necessary, which diminished the risk of injury to the sympathetic fibres of the gland. Other technical details are given in the text.

RESULTS

Electrical stimulation of the sympathetic trunk

Stimulation of the peripheral end of the cut sympathetic was found to cause a flow of saliva when a rate of stimulation well within the physiological range was used. Among fifteen cats studied, one responded with secretion at a rate of only 0·2/sec. In three cats 0·5/sec was found to be effective, in two others 1/sec; four responded at 2/sec, two at 3/sec, one at 4/sec and two at 5/sec.

When low frequencies were used a continuous secretion could be maintained. At a rate of 3–10/sec or more the flow diminished in spite of continued stimulation, and at rates of 10 or 20 sec the picture usually described as typical of sympathetically induced secretion was obtained: often the flow ceased completely within the first minute or minutes; sometimes a very slow flow persisted during the whole stimulation period; in some instances the flow ceased early, but periods of secretory activity reappeared now and again.

An attempt was made to find the cause of the decrease or cessation of the flow at high rates of stimulation. A reasonable explanation could be that the vasoconstriction simultaneously produced seriously interfered with the secretion, depriving the gland cells of blood necessary for the activity. It is well known, for instance, that a high rate of secretion cannot be maintained for a long period by chorda stimulation if the artery of the gland is partially clamped. It can, in fact, be seen that the secretory effect of sympathetic stimulation is more pronounced if the constriction is in some way counteracted. This can be achieved by stimulating the chorda after injection of atropine in a dose which entirely abolishes the secretory effect of parasympathetic stimulation.

Figure 1 shows such an experiment. Sympathetic stimulation caused secretion at a rapidly decreasing rate and a pronounced constriction. Chorda stimulation, after atropine, produced its marked vasodilatation but no secretion. When sympathetic stimulation was added, the flow of blood through the gland diminished to some extent, particularly initially; but it was much less reduced than was the case when the chorda was not stimulated. The secretory response to sympathetic stimulation was greater than before, suggesting that vasoconstriction might contribute to the decline in secretory rate. However, it could only be a contributing factor, for even in the period of increasing blood flow the secretion diminished and eventually ceased.

The falling off of the secretion at high rates of stimulation was observed even when the post-ganglionic sympathetic fibres running along the artery were stimulated, and could therefore not be due to failure of ganglionic transmission. A well-maintained secretion could, on the other hand, be elicited by continuous intravenous injection of adrenaline, and when secretion had ceased during sympathetic stimulation the injection of adrenaline was found to produce a flow of saliva. Consequently, the decline could not be attributed to some process in the glandular elements.

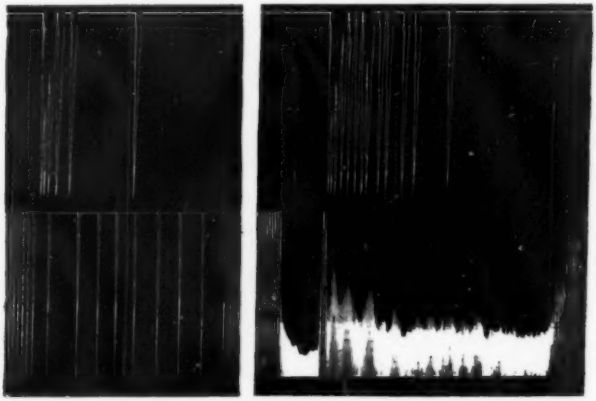


Fig. 1. Records from above down: drops of saliva; drops of blood from the gland; signal, which also marks time intervals of 10 sec. For secretion and blood flow, ordinate recorders were used, the upper one operated manually, the lower one by a phototube. In the left section of the tracing the effect of sympathetic stimulation for 5 min at a rate of 20/sec. Between the sections 2 mg atropine sulphate was given intravenously. In the right section the chorda was stimulated during the whole period marked by the signal (7 min). After 1 min, sympathetic stimulation was added and continued for 5 min.

It seems reasonable to assume that at the high rates of stimulation transmission for some reason failed at the endings of the post-ganglionic secretory fibres. This apparently did not occur at the vasoconstrictor nerve endings, for constriction persisted throughout the period of stimulation, as can be seen in Fig. 1. The only exception from this rule is that secretion may cause a temporary vasodilatation. In those cats in which secretion ceased initially but again appeared after some time, corresponding fluctuations in blood flow could be seen, as shown in the experiment of Fig. 2. Even in such cats, however, the secretion finally ceased definitely and constriction persisted.

Effect of restoring the resting vasomotor tone

In the experiment of Fig. 3 section of the sympathetic trunk caused an increased flow of blood through the gland. When the peripheral end of the trunk was stimulated electrically at a rate of 1/sec, a constriction was evoked slightly larger than that prevailing before the nerve was cut.

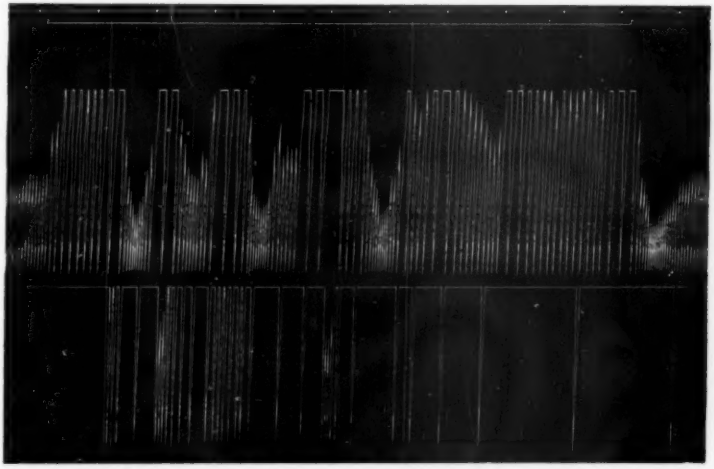


Fig. 2. Records from above down: time marker, minutes; signal, which marks the period of sympathetic stimulation at a rate of 20/sec; blood flow, and secretion; these were recorded as in Fig. 1.

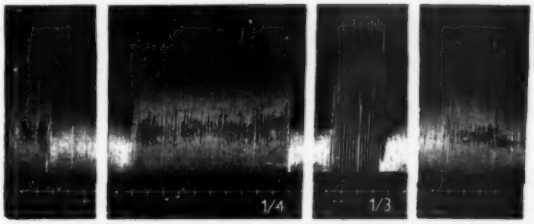


Fig. 3. From above down: blood flow through the gland; time marker, minutes; drops of saliva; signal. The first section of the tracing shows the effect of cutting the sympathetic trunk. In the next sections the peripheral end of the trunk was stimulated at different rates (1, 5 and 0·5/sec).

Already at this rate a slow secretion was obtained. Stimulation at 0·5/sec caused a smaller constriction but no secretion. This was a type of effect seen in most cats; but there were some experiments in which secretion was observed at a rate which was too small to restore the sympathetic resting

constrictor tone. It should be added that there is never any secretion normally which can be attributed to the resting constrictor activity.

Effect of a reflexly induced strong vasoconstriction

Figure 4 shows an experiment in which a pronounced vasoconstriction was evoked in the gland by bleeding the cat. It can be seen that the two main technical difficulties of these experiments had been overcome in this

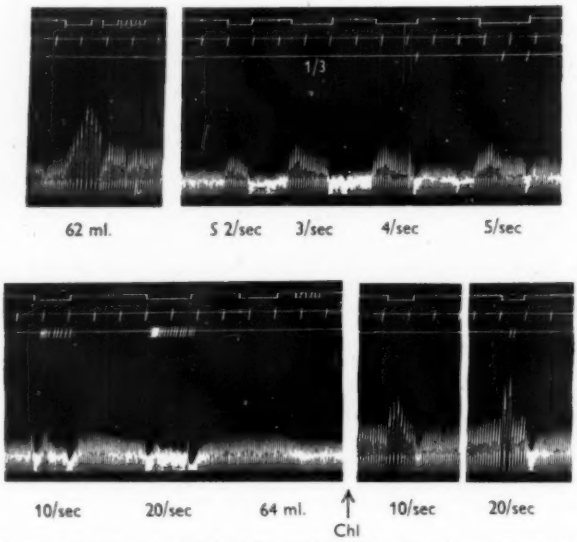


Fig. 4. From above down: signal; time marker, minutes; drops of saliva; blood flow through the gland, which was separately perfused from another cat. The first section shows the effect of withdrawal of blood from the recipient and the reinjection of the blood about 1 min later. This procedure was repeated in section 3. Between the first two sections the cervical sympathetic was cut. S = stimulation of its peripheral stump at different frequencies (2, 3, 4, 5, 10, 20/sec). Chl, chlorpromazine 0·4 mg/kg injected into the donor 30 min before the last section.

preparation. No vascular connexions had been left untied between the gland circulation and the remaining circulatory system of the recipient. This was obvious from the fact that bleeding did not alter the flow of blood from the gland when the cervical sympathetic had been severed. Further, the sympathetic innervation had not been destroyed in the course of the dissection, for stimulation of the sympathetic trunk produced the characteristic responses from the gland.

As is illustrated in Fig. 4, it is possible to produce a marked vasoconstriction in the gland reflexly via the sympathetic vasoconstrictor fibres, without any concomitant secretion. Electrical stimulation of the

sympathetic at a rate which causes a much smaller vasoconstriction is followed by secretion of saliva. It is, in fact, often difficult to reproduce the marked reflex vasoconstriction by artificial stimulation of the sympathetic, since the secretion obtained tends to produce a vasodilatation, obscuring the constrictor response. Attempts were made to get a pure constrictor response by abolishing the secretory effect of sympathetic stimulation. This can be attained by injecting a small dose of chlorpromazine (Emmelin, 1955). In the experiment of Fig. 4 this attempt was only partly successful. When the nerve was stimulated after chlorpromazine at a rate of stimulation of 10/sec, no secretion and a relatively pure constrictor response were obtained; before giving chlorpromazine the dominating vasomotor effect at this rate was vasodilatation. The constriction elicited after injection of the drug was somewhat smaller than that produced by bleeding. When the rate of stimulation was raised to 20/sec, a larger constriction appeared, but it was of short duration, presumably since a small secretion started. Judging from the size of the constriction obtained after chlorpromazine the impulse rate in the sympathetic during bleeding might have been of the order of 10–20/sec.

These experiments indicate that there is no spread of transmitter from the vasoconstrictor endings to the gland cells sufficient to evoke secretion, even if the constrictor fibres are intensely activated reflexly. They do not, however, exclude the possibility that some diffusion of the transmitter may take place. In order to test this possibility we have tried to make the gland cells particularly sensitive to secretory agents. This was achieved by cutting the chorda tympani some weeks before the acute experiment. In one experiment a particularly high supersensitivity had been created in this way. The gland cells responded to adrenaline 0·5 μg/kg intravenously instead of normally about 15 μg/kg (Emmelin & Muren, 1951). When a vasoconstriction was elicited in this cat by bleeding, a very slow secretion started after about 1 min; this effect was abolished by section of the sympathetic trunk in the neck.

DISCUSSION

When the cervical sympathetic trunk is stimulated electrically at low frequencies, a secretion is obtained which differs from that usually described in the literature in being continuous. The normal resting activity of the sympathetic on the blood vessels seems to be of an order of magnitude comparable to that which causes secretion on artificial stimulation; but it is not accompanied by secretion. According to Folkow (1955) normal resting vasoconstrictor tone is maintained at a rate of about 1–3 impulses/sec. When the cervical sympathetic is stimulated electrically at this rate, a secretion of saliva is obtained in most cats. Even when the

vasoconstrictor fibres are activated reflexly at a rate which probably approaches the limit of what is possible in the course of physiological events, no secretion is elicited in a normal gland. These observations indicate that specific secretory nerve fibres for the submaxillary gland of cats are present in the cervical sympathetic trunk. They do, of course, not exclude the possibility that transmitter diffusing from the terminals of the vasoconstrictor nerves might contribute to some extent to the secretory response provoked by the sympathetic stimulation, at least when the activity in the vasomotor fibres is high. The observations on the sensitized gland support this view.

SUMMARY

1. Secretion of saliva can be elicited from the submaxillary gland of cats by stimulating the cervical sympathetic trunk at a rate which is well within physiological limits.

2. If the sympathetic vasoconstrictor tone in the gland is abolished by section of the sympathetic in the neck, it can be restored by stimulating the peripheral end of the nerve trunk electrically at a rate of stimulation which in some cats may cause secretion; in others a slightly higher rate is required to cause a flow of saliva.

3. Even when the vasoconstrictor fibres of the gland are strongly activated by bleeding, no secretion is obtained in a normal gland, although the impulse rate in the constrictor fibres must by far exceed the secretory threshold rate for artificial electrical stimulation.

4. These observations indicate that the cervical sympathetic trunk contains specific secretory fibres for the submaxillary gland.

REFERENCES

CANNON, W. B. & ROSENBLUETH, A. (1937). *Autonomic Neuro-effector Systems.* New York: MacMillan.

CELANDER, O. (1959). Are there any centrally controlled sympathetic inhibitory fibres to the musculature of the intestine? *Acta physiol. scand.* **47**, 299–309.

EMMELIN, N. (1955). Sympathicolytic agents used to separate secretory and vascular effects of sympathetic stimulation in the submaxillary gland. *Acta physiol. scand.* **34**, 29–37.

EMMELIN, N. & ENGSTRÖM, J. (1959). Sympathetic secretory fibres for the cat's submaxillary gland. *J. Physiol.* **149**, 67–68*P*.

EMMELIN, N. & MUREN, A. (1951). Sensitization of the submaxillary gland to chemical stimuli. *Acta physiol. scand.* **24**, 103–127.

FOLKOW, B. (1955). Nervous control of the blood vessels. *Physiol. Rev.* **35**, 629–663.

KOCK, N. G. (1959). An experimental analysis of mechanisms engaged in reflex inhibition of intestinal motility. *Acta physiol. scand.* **47**, Suppl. 164, 1–54.

KUNTZ, A. & RICHINS, C. A. (1946). Components and distribution of the nerves of the parotid and submandibular glands. *J. comp. Neurol.* **85**, 21–32.

RICHINS, C. A. & KUNTZ, A. (1953). Role of sympathetic nerves in the regulation of salivary secretion. *Amer. J. Physiol.* **173**, 471–473.

SIMEONE, F. A. & MAES, J. P. (1939). Sensitization of submaxillary gland by sympathetic denervation. *Amer. J. Physiol.* **125**, 674–679.

J. Physiol. (1960), **153**, *pp.* 9–16
With 3 *text-figures*
Printed in Great Britain

EFFECT OF SYMPATHETIC DENERVATION ON THE SENSITIVITY OF THE SUBMAXILLARY GLAND TO STIMULATING AGENTS

BY N. EMMELIN AND J. ENGSTRÖM
From the Institute of Physiology, University of Lund, Sweden

(*Received* 13 *January* 1960)

Numerous investigations have dealt with the supersensitivity of salivary glands which develops after section of the parasympathetic secretory fibres. Much less attention has been paid to the effect of destruction of the sympathetic fibres of the glands. Simeone & Maes (1939) studied the secretory action of adrenaline, acetylcholine and pilocarpine on the submaxillary gland of cats 47–90 days after excision of the superior cervical ganglion of one side. A moderate supersensitivity towards these drugs was found in most of the cats; in some no sensitization had occurred. Similar observations were reported by Emmelin & Muren (1951). They found in addition a supersensitivity to noradrenaline which was somewhat more pronounced and was obtained more regularly than that seen when adrenaline was used as a test drug; this was also observed by Strömblad (1956). Dirnhuber & Lovatt Evans (1954) found a supersensitivity to adrenaline but none towards acetylcholine a few weeks after removal of the ganglion. In all these investigations the level of sensitivity was estimated in an acute experiment, the gland of the unoperated side serving as a control.

In the present experiments the effect of removal of the superior cervical ganglion on the sensitivity of the gland was estimated by a method which permits repeated observations in one and the same cat over long periods. The development of a supersensitivity could therefore be studied. In some of these cats the preganglionic sympathetic fibres were first severed; it is not known whether such an operation affects the sensitivity of the gland. For a comparison the responses of the nictitating membrane to adrenaline and noradrenaline were recorded simultaneously. Preganglionic denervation is known to increase the sensitivity of the membrane; post-ganglionic denervation has an even larger sensitizing effect (Hampel, 1935).

In addition, the effect was investigated of removal of the sympathetic ganglion on the sensitivity of the gland cells towards impulses in the parasympathetic secretory fibres. This was done in acute experiments, the ganglion of one side having been excised some weeks in advance.

METHODS

In order to make repeated observations on the sensitivity of the gland the following method was used (Emmelin & Muren, 1952). After preliminary ether anaesthesia a short-acting barbiturate was given intracardially. Secretory agents were injected by the same route, and between the injections saline solution was slowly infused to prevent clotting in the injection needle. The submaxillary ducts were cannulated from the mouth with fine glass cannulae. The drops of saliva which fell were counted. The salivary cannulae and the needle in the heart were then removed, the cat was allowed to wake up and the experiment could be repeated after some days. The drugs tested were adrenaline, noradrenaline, cobefrin, acetylcholine, and methacholine. When only the sympathomimetic amines were given to evoke secretion, hexobarbitone (Evipan; Bayer Products) was employed as an anaesthetic as in the original method. In the doses used (about 20 mg/kg initially, with repeated smaller doses when required) this barbiturate does not affect the sensitivity of the gland towards these secretory agents; it exerts, however, an atropine-like action. Thiobarbiturates are known to lack this effect (Gruber, Gruber & Colosi, 1938). Two different thiobarbiturates were tried. Thiopentone (Pentothal; Abbot Laboratories) proved satisfactory but the best results were obtained with Baytinal Bayer (Sodium 5, 5-allyl-(2′-methyl-propyl)-thiobarbituricum). These barbiturates interfered neither with the secretory action of the parasympathomimetic nor with that of the sympathomimetic agents. The initial doses of thiopentone and Baytinal were 5 and 20 mg/kg, respectively. Before the injection of acetylcholine or methacholine, dihydroergotamine (0·5 mg) was given intracardially to abolish any secretory effect due to catechol amines which could have been released from the adrenals or from the endings of the sympathetic secretory fibres.

Contractions of the nictitating membrane in response to adrenaline and noradrenaline were recorded under standard conditions. A fine clip was fixed to the margin of the membrane and connected to a lever by a thread and pullies.

The preganglionic trunk was cut or the superior cervical ganglion excised in an aseptic operation in barbiturate anaesthesia.

In order to study the effect of unilateral removal of the ganglion on the sensitivity towards impulses sent to the gland cells through the parasympathetic fibres, cats were anaesthetized with chloralose and both submaxillary ducts cannulated. The chordolingual nerve was cut on both sides and the peripheral stumps excited electrically by stimulation at different low frequencies.

RESULTS

Removal of the ganglion

The effect on the gland of extirpation of the superior cervical ganglion was examined in nineteen cats. The sensitivity was expressed as number of drops of saliva secreted in response to a series of standard doses of the test drugs (noradrenaline, 1, 2, 5, 10, 20 μg/kg; adrenaline, 0·5, 1, 2, 5, 10, 20 μg/kg; cobefrin, 10, 20, 40 μg/kg; acetylcholine, 0·5, 1, 2, 5 μg/kg; methacholine, 0·5, 1, 2 μg/kg).

The results of these experiments are summarized in Table 1. It can be seen that a supersensitivity to the various drugs was obtained in most instances. Towards noradrenaline a sensitization was seen in all the ten cats studied; with acetylcholine, on the other hand, the sensitization was much less regular. Acetylcholine and methacholine were tried in the same

twelve cats; it can be seen that three of these showed a supersensitivity towards the latter but not to the former drug. Like earlier investigators we observed that the most pronounced sensitization was detected with noradrenaline (Figs. 1, 2); with methacholine the effect was also often marked. Even with these drugs, however, the level of supersensitivity attained was not more than moderate, and usually far below that seen after para-

TABLE 1

Drug tested	No. of cats studied	No. of cats showing supersensitivity	No. of cats with no definite supersensitivity
Noradrenaline	10	10	0
Adrenaline	19	13	6
Cobefrin	4	4	0
Acetylcholine	12	7	5
Methacholine	12	10	2

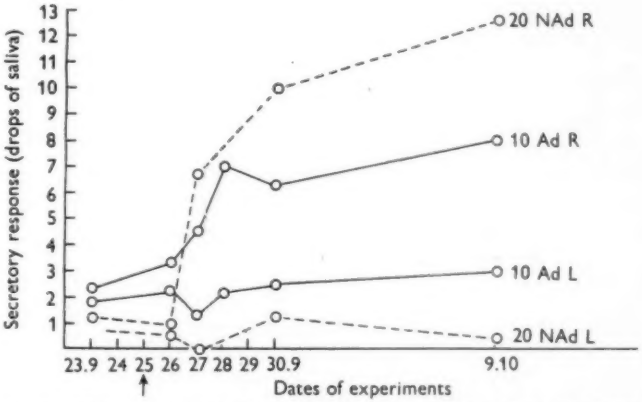


Fig. 1. Sensitivity of the right (R) and left (L) submaxillary gland to adrenaline 10 μg/kg and noradrenaline 20 μg/kg. At the arrow the right superior cervical ganglion was extirpated.

sympathetic denervation of the gland (see, for instance, Emmelin & Muren, 1951). It was also characteristic that the degree of sensitization varied very much in different cats.

In those cats in which supersensitivity was obtained, it was found regularly, each time the secretory responses to drugs were examined. Sensitization could first be detected 1–2 days after excision of the ganglion, and the sensitivity increased rapidly in the course of the following days. The development of the supersensitivity is illustrated in Fig. 1; in order to show this clearly an experiment was chosen in which a particularly pronounced supersensitivity towards noradrenaline and adrenaline was

obtained. The supersensitivity then remained; in three cats it was seen as late as 10 months after denervation.

Pre- and post-ganglionic denervation

Figure 2 demonstrates the effects of pre- and post-ganglionic sympathetic denervation on the submaxillary gland and the nictitating membrane. After preganglionic section the membrane showed its well-known supersensitivity, both to noradrenaline and adrenaline; this was further increased by removal of the ganglion. The sensitivity of the gland did not

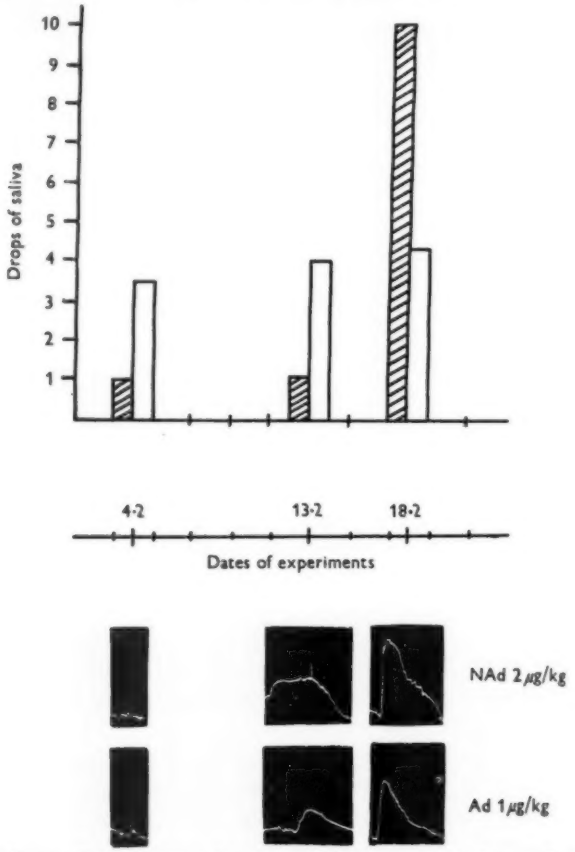


Fig. 2. Above, effects of pre- and post-ganglionic sympathetic denervation on the submaxillary gland; and below, on the nictitating membrane. Preganglionic denervation was carried out on February 4, post-ganglionic on February 13. ▨, NAd 10 μg/kg; □, Ad, 5 μg/kg.

increase after preganglionic section. When the ganglion had been excised the sensitivity towards noradrenaline had greatly increased. In this particular cat there was no sensitization to adrenaline after ganglionectomy, in others there was. A regular finding, however, was that preganglionic sympathetic denervation did not sensitize the gland to the two drugs.

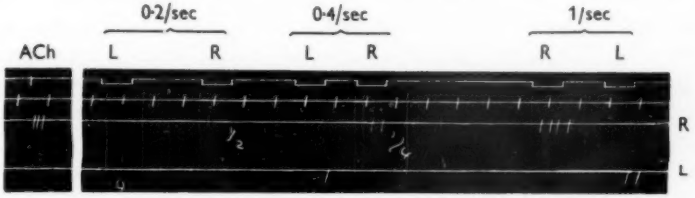


Fig. 3. Experiment in chloralose anaesthesia. Secretory effects of acetylcholine 2 μg/kg (ACh), given intravenously, and of stimulation of the chorda tympani for 1 min at a rate of 0·2, 0·4, and 1/sec. R = right gland, L = left gland. The right superior cervical ganglion had been excised 23 days earlier.

Sensitization to parasympathetic stimulation

When a low-frequency stimulation of the chorda was used, a supersensitivity towards chorda impulses could be seen after removal of the sympathetic ganglion. This is shown in the experiment of Fig. 3. At a rate of 0·2/sec a secretory response was evoked from the denervated gland only (half a drop of saliva); at 0·4 and 1/sec the responses of this gland were larger than those of the normal gland.

DISCUSSION

The experiments show that it is possible to produce a supersensitivity in the submaxillary gland by removal of the superior cervical ganglion, regularly towards noradrenaline, less regularly towards some other agents. Variations in the degree of supersensitivity in different cats probably reflect the fact that the sympathetic innervation seems to vary in extent; in some cats a profuse secretion is obtained on sympathetic stimulation, in others the response is small and may even be absent.

The sensitization obtained, although less pronounced, resembles very much that caused by section of the parasympathetic secretory fibres. This fact may be quoted as an argument in favour of the view, expressed in the preceding paper (Emmelin & Engström, 1960), that the sympathetic contains secretory fibres.

The sensitization towards cofebrin is of particular interest, since this drug is not destroyed by amine oxidase (Blaschko, 1954). This observation supports the conclusion of Strömblad (1956) that amine oxidase is not involved in the process of sensitization after ganglionectomy.

It has been suggested that the level of sensitivity of the gland is to a great extent dependent on the flow of secretory impulses from the central nervous system (Emmelin & Muren, 1950). The chorda tympani is the main secretory nerve of the gland; in fact, no digestive salivary reflexes seem to be mediated via the sympathetic pathway. This could explain why a marked supersensitivity follows when the gland is disconnected from the central nervous system by section of the preganglionic parasympathetic fibres, whereas no supersensitivity ensues after section of the preganglionic sympathetic fibres. It is of interest in this connexion to point out that this latter operation sensitizes the nictitating membrane, as was shown by Hampel (1935); the membrane usually is kept in a state of contraction by impulses from the central nervous system. In support of the view that section of the sympathetic trunk causes no supersensitivity in the gland because the gland is not deprived of any impulses, the following observations may be quoted. The supersensitivity caused by section of the chorda can be greatly diminished if the gland is supplied with impulses from the hypoglossal nerve; this occurs not only if the hypoglossal nerve is connected in a crossed anastomosis to the parasympathetic pathway, but also if it is made to regenerate into the sympathetic trunk (Emmelin, Muren & Strömblad, 1957*a*, *b*).

The fact that a supersensitivity is produced by removal of the sympathetic ganglion suggests that the post-ganglionic sympathetic neurone exerts some action on the gland which is not due to impulses from the central nervous system. Such an influence has been attributed to the post-ganglionic parasympathetic neurone; this conception was based mainly on the fact that prolonged treatment with a parasympatholytic agent causes a greater supersensitivity than section of the preganglionic parasympathetic fibres (Emmelin & Strömblad, 1957; Emmelin, 1960). In this connexion it may be pointed out that Burn & Rand (1959) attribute the supersensitivity which they observe in smooth muscle after sympathetic denervation or treatment with reserpine to the disappearance of a store of noradrenaline.

The two sets of autonomic secretory nerves are generally assumed to act on different types of gland cells (see Babkin, 1950). Langenskiöld (1941), however, came to the conclusion that they innervate the same cells, and support of this view has been given later (Emmelin, 1955; Lundberg, 1955). The finding that removal of the sympathetic ganglion can sensitize the gland cells towards impulses of low frequency in the parasympathetic nerve seems to afford additional evidence in support of this conception.

SUMMARY

1. The sensitivity of the submaxillary gland of cats to chemical agents was estimated in repeated experiments in one and the same animal, anaesthetized with a short-acting barbiturate. At the same time the sensitivity of the nictitating membrane was determined.

2. Preganglionic sympathetic denervation, sensitizing the membrane, did not change the responsiveness of the salivary gland. After removal of the superior cervical ganglion both structures acquired an increased sensitivity. In the gland the supersensitivity was particularly marked towards noradrenaline; the sensitivity towards adrenaline, acetylcholine and methacholine was not always augmented.

3. Supersensitivity could first be observed after 1–2 days, and it increased rapidly in the course of the following days to remain at a high level for long periods, up to 10 months. The degree of sensitization varied in different cats. It was usually much less than that produced by section of the chorda tympani.

4. When the sympathetic ganglion had been removed in advance, the gland was found to be supersensitive to impulses in the chorda tympani.

REFERENCES

BABKIN, B. P. (1950). *Secretory Mechanism of the Digestive Glands*, 2nd ed. New York: Hoeber.

BLASCHKO, H. (1954). Metabolism of epinephrine and norepinephrine. *Pharmacol. Rev.* **6**, 23–28.

BURN, J. H. & RAND, M. J. (1959). The cause of the supersensitivity of smooth muscle to noradrenaline after sympathetic degeneration. *J. Physiol.* **147**, 135–143.

DIRNHUBER, F. & LOVATT EVANS, C. (1954). The effects of anticholine-sterases on humoral transmission in the submaxillary gland. *Brit. J. Pharmacol.* **9**, 441–458.

EMMELIN, N. (1955). On the innervation of the submaxillary gland cells in cats. *Acta physiol. scand.* **34**, 11–21.

EMMELIN, N. (1960). Is there a leakage of acetylcholine from postganglionic parasympathetic nerve endings? *Nature, Lond.*, **185**, 297–298.

EMMELIN, N. & ENGSTRÖM, J. (1960). On the existence of specific secretory sympathetic fibres for the cat's submaxillary gland. *J. Physiol.* **153**, 1–8.

EMMELIN, N. & MUREN, A. (1950). Supersensitivity of denervated organs to chemical stimuli. *Nature, Lond.*, **166**, 610.

EMMELIN, N. & MUREN, A. (1951). Sensitization of the submaxillary gland to chemical stimuli. *Acta physiol. scand.* **24**, 103–127.

EMMELIN, N. & MUREN, A. (1952). The sensitivity of submaxillary glands to chemical agents studied in cats under various conditions over long periods. *Acta physiol. scand.* **26**, 221–231.

EMMELIN, N., MUREN, A. & STRÖMBLAD, R. (1957*a*). Effect of anastomosis between the hypoglossal and chorda-lingual nerves on the supersensitivity of the denervated submaxillary gland. *Acta physiol. scand.* **41**, 18–34.

EMMELIN, N., MUREN, A. & STRÖMBLAD, R. (1957*b*). Effect of anastomosis between the hypoglossal and sympathetic nerves on the supersensitivity of the denervated submaxillary gland. *Acta physiol. scand.* **41**, 35–48.

EMMELIN, N. & STRÖMBLAD, B. C. R. (1957). Sensitization of the submaxillary gland above the level reached after section of the chorda tympani. *Acta physiol. scand.* **38**, 319–330.

GRUBER, C. M., GRUBER, C. M. JR. & COLOSI, N. A. (1938). The irritability of the cardiac vagus nerves as influenced by the intravenous injections of barbiturates, thio-barbiturates and picrotoxin. *J. Pharmacol.* **63**, 215–228.

HAMPEL, C. W. (1935). The effect of denervation on the sensitivity to adrenine of the smooth muscle in the nictitating membrane of the cat. *Amer. J. Physiol.* **111**, 611–621.

LANGENSKIÖLD, A. (1941). Component potentials of the submaxillary gland electrogram and their relation to innervation and secretion. *Acta physiol. scand.* **2**, Suppl. 6, 1–109.

LUNDBERG, A. (1955). The electrophysiology of the submaxillary gland of the cat. *Acta physiol. scand.* **35**, 1–25.

SIMEONE, F. A. & MAES, J. P. (1939). Sensitization of the submaxillary gland by sympathetic denervation. *Amer. J. Physiol.* **125**, 674–679.

STRÖMBLAD, B. C. R. (1956). Supersensitivity and amine oxidase activity in denervated salivary glands. *Acta physiol. scand.* **36**, 137–153.

J. Physiol. (1960), **153**, *pp.* 17–34
With 8 *text-figures*
Printed in Great Britain

THE ACTION OF PROCAINE AND ATROPINE ON SPINAL NEURONES

BY D. R. CURTIS AND J. W. PHILLIS

From the Department of Physiology, Australian National University, Canberra, Australia

(*Received* 22 *February* 1960)

Procaine has a curare-like action at the end-plate region of muscle, reducing the effectiveness of synaptically released and iontophoretically applied acetylcholine (Harvey, 1939; del Castillo & Katz, 1957). Furukawa (1957) has also demonstrated a direct effect upon the spike-generating mechanism of muscle fibres, an observation which is in accordance with the more recent findings of the action of procaine on nerve fibres (Shanes, Freygang, Grundfest & Amatniek, 1959; Taylor, 1959).

It has been reported that, when applied iontophoretically to Renshaw cells in the mammalian spinal cord, procaine depressed their activation by acetylcholine (Curtis & Eccles, 1958*b*). This observation led readily to the assumption that procaine was preventing the access of acetylcholine to the subsynaptic receptors of these cells. However, procaine may also have a more direct action upon the membrane of Renshaw cells; consequently an investigation was carried out in order to determine its effect upon both cholinoceptive Renshaw cells and non-cholinoceptive interneurones and motoneurones. In addition, a study was made of the action of atropine, another compound which blocks the action of acetylcholine and which possesses, moreover, certain structural similarities to cocaine.

METHODS

All methods have been reported in previous publications (Curtis & Eccles, 1958*a*; Curtis, Phillis & Watkins, 1959, 1960*a*). Motoneurones, interneurones and Renshaw cells of the lower lumbar segments of the spinal cord of the cat were identified by their responses to volleys entering the spinal cord via the dorsal and ventral roots. The cord was transected at the lower thoracic level, the animal being lightly anaesthetized with pentobarbital sodium.

Procaine and atropine were passed iontophoretically as cations from saturated aqueous solutions of procaine hydrochloride and atropine sulphate respectively in double, five-barrel or co-axial electrodes. The double and five-barrel electrodes permitted the extracellular recording of spike potentials of neurones, whereas the co-axial electrode assembly allowed the simultaneous recording of intracellular responses of motoneurones whilst applying these substances extracellularly (cf. Curtis *et al.* 1959). Other substances used in this investigation were acetylcholine, which was passed iontophoretically as a cation from an aqueous solution of acetylcholine bromide, and glutamic acid, which was passed as an anion from a solution

in NaOH at pH 8 (cf. Curtis *et al.* 1960*a*). These substances were prevented from diffusing out of the electrodes by the application of suitable backing potentials. When co-axial electrodes were used, intracellular spike potentials recorded by the inner electrode were considerably attenuated owing to the high input-capacitance of the recording system. Cells impaled by these electrodes usually had resting potentials in the range -45 to -55 mV, in comparison with those of -60 to -70 mV found when single-barrel electrodes were used (cf. Coombs, Eccles & Fatt, 1955). These low potentials were presumably due to damage caused by the electrode. However, such potentials could be maintained for periods of 30 min–1 hr and the responses to both procaine and atropine resembled those given by cells with high resting potentials.

RESULTS

Procaine

It has been demonstrated that Renshaw cells, when fired by ventral root volleys, are activated synaptically by acetylcholine (Curtis & Eccles, 1958*b*). The depressant action of procaine upon this firing has been confirmed. Renshaw cells are also excited in a repetitive manner by the anions of certain of the acidic amino acids, in particular glutamic, aspartic and cysteic acids (Curtis *et al.* 1960*a*). It has been postulated that this excitation is mediated through receptors other than those specialized for acetylcholine, because dihydro-β-erythroidine (DHE), although effectively blocking the cholinergic excitation of Renshaw cells, is without action upon the firing by these excitant amino acids. Unlike DHE, procaine blocks the excitation of Renshaw cells produced by the excitant amino acids. The Renshaw cell which was excited by a ventral root volley in Fig. 1A also responded to acetylcholine (Fig. 1B) and glutamate ion (Fig. 1C). These responses were recorded by means of the central barrel of a five-barrel electrode. One minute after a current of 100 nA began to pass procaine from another barrel of the electrode, the same types of stimulation were applied. The response to the ventral root stimulus (D) was partially blocked, and the responses to acetylcholine (E) and glutamate ion (F) were completely suppressed. Two minutes after the cessation of the procaine application, almost complete recovery had occurred, G, H and I corresponding to A and D, B and E, C and F respectively.

These results might suggest that, as glutamate ion excited these cells without attachment to acetylcholine receptors, procaine may not only block such cholinoceptive sites but also the 'amino-acid receptors'. This postulate is unlikely, owing to the structural differences between the two series of excitant substances, and it is therefore possible that procaine has a more direct action on those processes producing the spike potential. This latter postulate could be tested by applying procaine to interneurones and motoneurones of the spinal cord, for these cells have no cholinoceptive synapses (Curtis, D. R., Phillips, J. W. and Watkins, J. C.—unpublished observations). It was found that the spikes evoked by both orthodromic

stimulation and the excitant amino acids were blocked by procaine. The responses of Fig. 2A–D were recorded from an interneurone lying in the dorsal horn of the seventh lumbar segment, in response to a single maximal stimulus applied to the ipsilateral peroneal nerve. These extracellular records were obtained by using the NaCl-containing barrel of a double-barrel elec-

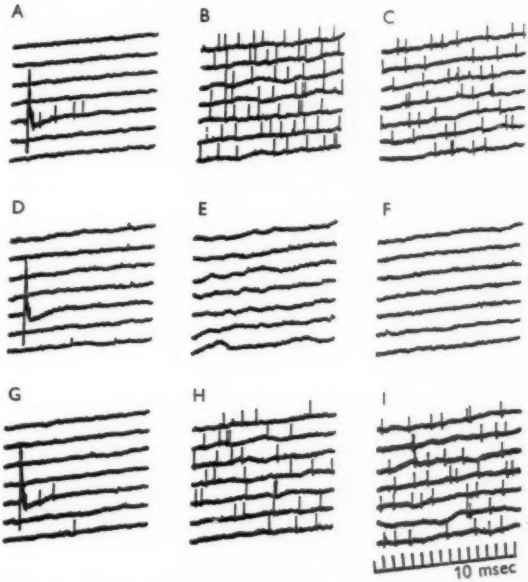


Fig. 1. Spike potentials recorded extracellularly from a Renshaw cell by means of the central barrel of a five-barrel electrode and evoked by a ventral root stimulus A, D and G; by iontophoretic application of acetylcholine, B, E and H; and by iontophoretic application of glutamic acid, C, F and I. The iontophoretic currents used in each of the two series of applications were constant throughout. After the control responses A–C, procaine was passed as a cation from another barrel of the electrode and the responses D, E and F were recorded during this application. G, H and I were recorded 2 min after the procaine application ceased. These responses were recorded on film moving parallel to the Y axis of the oscilloscope, there being less than 5 msec between sweeps. Time marker, 10 msec.

trode, and typically the spikes were repetitive (Fig. 2A). Following the application of procaine by means of a cationic current of 135 nA through the procaine-containing barrel of the electrode, the spikes were slowly reduced in size and number. The records (B) and (C) were taken 15 and 40 sec respectively after the commencement of this current. The effect was fully reversible, as is illustrated in Fig. 2D, which was recorded 20 sec after termination of the iontophoretic application. This action was not due to the flow of current

through the electrode, for the spike height of the cell was not altered by the passage of even greater currents through the NaCl-containing barrel, by means of which the responses were recorded, and currents in the reverse direction through the procaine-containing barrel did not increase the size or number of spikes. In some cells similar applications of procaine led to a reduction in the number of spikes without altering spike size. Usually, however, a diminution in spike size as well as number was observed.

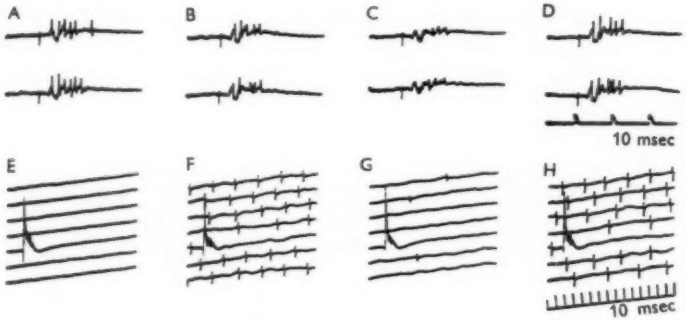


Fig. 2, A–D. Extracellular spike potentials of an interneurone located in the dorsal horn of the seventh lumbar segment and evoked by maximal stimulation of the peroneal nerve, recorded by means of one barrel of a double electrode. A, control responses; B and C, 15 and 40 sec after a current of 135 nA began to pass procaine from the other barrel of the electrode, this current flowing for 60 sec; D, 20 sec after the iontophoretic current ceased. Time marker, 10 msec.
E–H. Spikes of a similar cell but from another preparation, recorded in the same manner as those of Fig. 1. E, stimulation of the seventh lumbar dorsal root, this stimulus being repeated in F, G and H; F, application of glutamic acid as an anion; G, as for F but 48 sec after a current of 100 nA began to pass procaine from another barrel of the electrode; H, as for F but 12 min after the application of procaine ceased. Time marker, 10 msec.

The control responses of Fig. 2E and F were produced by stimulation of the seventh lumbar dorsal root and iontophoretic application of glutamate ion respectively. They were recorded by the central barrel of a five-barrel electrode. A cationic current of 100 nA then passed procaine from another barrel of the electrode, and Fig. 2G shows that not only was glutamate ion relatively ineffective thereafter, but also that the orthodromic spikes were reduced in number and size. Again full recovery was observed in Fig. 2H, these responses being recorded 12 min after the current applying procaine was terminated. These effects of procaine were observed upon spikes evoked by either aspartate, glutamate or cysteate ions and were not due to the interference between iontophoretic currents applying substances of opposite charge (cf. Curtis *et al.* 1959), since the observed action of

procaine often persisted for many minutes after the cationic current applying it had ceased.

In addition, procaine was passed iontophoretically around motoneurones, from which intracellular records were being obtained. Co-axial electrodes were used for these experiments and the results from several cells are illustrated in Figs. 3 and 4. Figure 3G represents the experimental situation with the inner barrel of the electrode in an intracellular position, whereas the outer barrel was located extracellularly with respect to the motoneurone (MN). In most cases no action was observed on the resting potential of the cell when procaine was applied iontophoretically, although in a few cells a small hyperpolarization of 1–2 mV was observed. In spite of this virtual absence of change in the resting potential, the spike potentials of motoneurones, whether elicited by synaptic, antidromic or direct excitation, were altered considerably. In all cells from which records were obtained the intracellular electrode was recording from the soma–dendritic region (cf. Coombs, Curtis & Eccles, 1957). Thus the full spike potential consisted of the spikes produced in a sequential fashion from both the initial segment (IS) and the soma–dendritic region (SD). In spite of resting potentials in the range -50 to -60 mV, the spikes were usually much less than 50 mV in height, owing to the distortion produced by the recording system. However, spike size was not an important consideration in the assessment of results. The responses of Fig. 3A–F were obtained from a motoneurone which was fired directly (ii) by a rectangular current pulse passed through the central barrel of the electrode, the size of this current pulse being indicated by the upper trace (i). In this manner the direct excitability of the membrane could be tested, the pulse size being altered under the varying conditions in order to straddle the threshold for excitation. Thus the size of the pulse indicated that current which was necessary to excite the cell in about half of 6–8 tests. Potentials generated by current flow through the electrode (see Fig. 3C, (ii)) were partially balanced out by a bridge circuit (Coombs, Curtis & Eccles, 1959). In addition, the axon of this cell was stimulated in the ventral root, the lower trace (iii) indicating the antidromic spike potential, which had an inflexion upon its rising phase due to the delay between transmission of the impulse from the IS to SD regions. Figure 3A is a control response; B and C were recorded 90 and 110 sec after a current of 400 nA commenced to pass procaine into the environment of the cell for a total duration of 115 sec. Following the cessation of this current and the re-application of a backing current of 200 nA in the reverse direction, the responses of Fig. 3D, E and F were recorded 25, 40 and 75 sec later. As indicated by the lower trace (iii), antidromic invasion of the SD segment was blocked (C and D) after a period in which invasion did not occur with every stimulus (B).

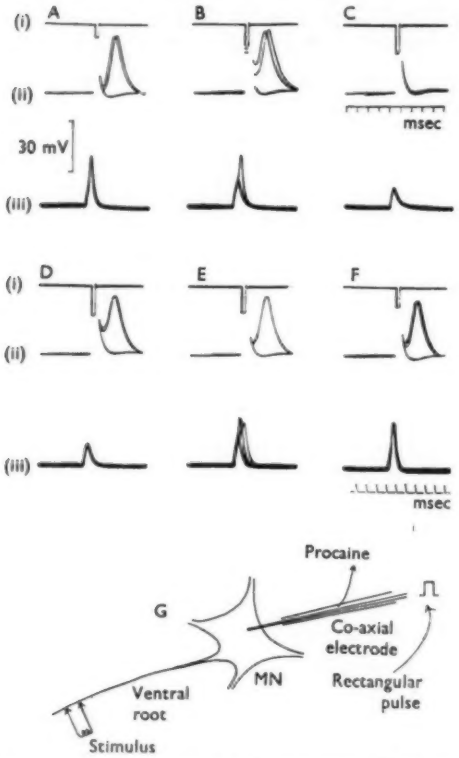


Fig. 3, A–F. Responses recorded from a motoneurone (MN) by means of the inner K_2SO_4-containing barrel of a co-axial electrode, as illustrated in G. The outer barrel contained a saturated solution of procaine hydrochloride from which procaine was applied as a cation for 115 sec after the control responses A were recorded. The resting potential of this cell was −56 mV and increased to −57 mV during the procaine application. Each record consists of six superimposed responses at a frequency of 1/sec, the antidromic and directly evoked spikes being recorded 500 msec apart.

A, control responses:

(i) Upper beam, relative magnitude of the current pulse which had a duration of 0·3 msec and evoked a spike in roughly half the tests.

(ii) Middle beam, spike potential evoked by a rectangular current pulse passed through the intracellular electrode.

(iii) Lower beam, spike potential evoked by stimulating the ventral root.

B–F, as for A except that the pulse size was altered in order to straddle the threshold for direct excitation. B, 90 sec; C, 110 sec after the commencement of the iontophoretic current; D, 25 sec; E, 40 sec and F, 75 sec after its termination. Time marker, msec for (i) and (ii) below middle beam of C; msec for the antidromic spike (iii) below F. Voltage, 30 mV for (ii) and (iii).

During recovery partial invasion of this segment occurred (E), but 75 sec after the procaine application had ceased, the SD segment was again being invaded by the spike potential (F). The direct threshold of the membrane was also increased by the action of procaine, as indicated by the size of the pulse (i) necessary to produce a full spike. In record C a pulse approximately 2·3 times the size of the control (A) failed to elicit a spike, but in F, after the cessation of the procaine, the spike was produced by a pulse 1·5 times the size of the control. Complete recovery occurred after a further period of about 30 sec. These changes were accompanied by a hyperpolarization of 2 mV, which is insufficient by itself to alter the direct excitability of the cell by a factor of 2·3.

Figure 4 illustrates responses from two other motoneurones to which procaine was applied iontophoretically. The cell of Fig. 4A–F was excited orthodromically, the just-threshold excitatory post-synaptic potential (EPSP) being shown in Fig. 4A and the truncated spike potential in Fig. 4B, C, E and F. The responses were evoked by stimulation of the gastrocnemius nerve, the intensity of the stimulus producing the potentials of Fig. 4A being doubled for the remaining records of the series. A current of 200 nA passed procaine from the outer barrel of the electrode for 75 sec and the subsequent responses C–F were recorded 48, 72, 95 and 135 sec after the commencement of this current. In records C and D, taken during the application, there was a progressive failure of spike generation, the EPSP of D being in fact larger than that of A, and yet completely failing to evoke a spike. Recovery occurred after the termination of the iontophoretic current, the EPSP's of Fig. 4E, however, producing spikes at slightly higher levels of membrane potential than those of A. Thereafter (F) a full spike only was produced. This cell had a comparatively low resting potential of − 50 mV and a low threshold for excitation, the control value of the just-threshold EPSP in Fig. 4A being 2–3 mV. However, the results were typical of those from five other motoneurones and clearly indicate that the orthodromic activation of motoneurones is blocked, not by an action of procaine on the production of excitatory post-synaptic potentials, but by a more direct effect upon the membrane. Observations of inhibitory post-synaptic potentials (IPSP's) indicated that they also were unaltered by procaine. Thus, for the cell illustrated in Fig. 4G–I, each of the pairs of IPSP's and antidromic spike potentials were recorded virtually simultaneously, Fig. 4G being the control responses and Figs. 4H and I the responses during and after the iontophoretic application of procaine. The procaine effectively blocked the antidromic invasion of the soma–dendritic membrane (H) but failed to alter the size of the IPSP. This is in contrast to the action of the depressant amino acids (Curtis *et al.* 1959), which, although having an action similar to procaine upon the

antidromic invasion of the cell, also reduced the size of both IPSP's and EPSP's. Direct measurement of the resistance of the cell membrane was not attempted in this series of experiments. However, the observation

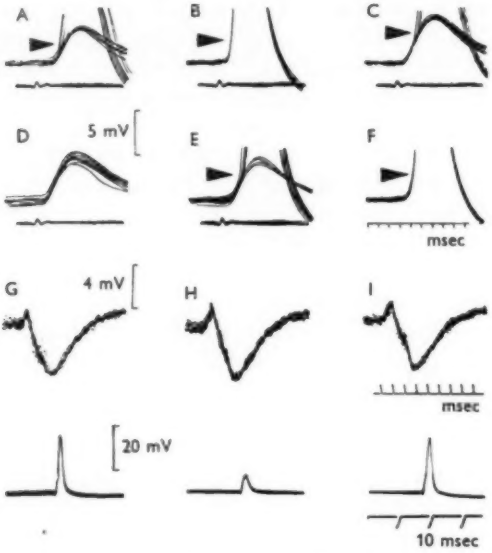


Fig. 4, A–F. Monosynaptic excitatory post-synaptic potentials recorded, as in Fig. 3, but from another motoneurone, in response to orthodromic stimulation, the volley arriving at the dorsal surface of the spinal cord indicated in the lower beam. The cell had a resting potential of −50 mV. Each figure consists of 15–20 superimposed records at a frequency of 5/sec.

A, B, control responses with maximal stimulation in B, whilst in A the EPSP just straddled the threshold of the cell. C–F, as for B but during and after the application of procaine to the cell by a current of 200 nA for 75 sec: C, 48 sec; D, 72 sec; E, 95 sec and F, 135 sec after this current commenced. The horizontal arrows indicate the size of the threshold EPSP.

G–I. Potentials similarly recorded from another motoneurone having a resting potential of −55 mV. The inhibitory potential (upper beam) was evoked by stimulation of the peroneal nerve whereas the antidromic spike potential (lower beam) was produced by stimulation of the segmental ventral root. G, control response; H, 60 sec after a current of 330 nA had been passing procaine from the outer barrel of the electrode; I, 70 sec after this current ceased.

that post-synaptic potentials were unchanged indicates that this resistance was altered but little by procaine. If the membrane conductance had been increased by procaine, the subsynaptic currents generating post-synaptic potentials would have been partially shunted, thereby reducing the

magnitude of the resultant potentials (cf. Eccles, 1957). In addition, the failure to reduce the size of these potentials indicates that impulse transmission in the presynaptic terminals was not impaired by the concentrations of procaine obtained in these experiments.

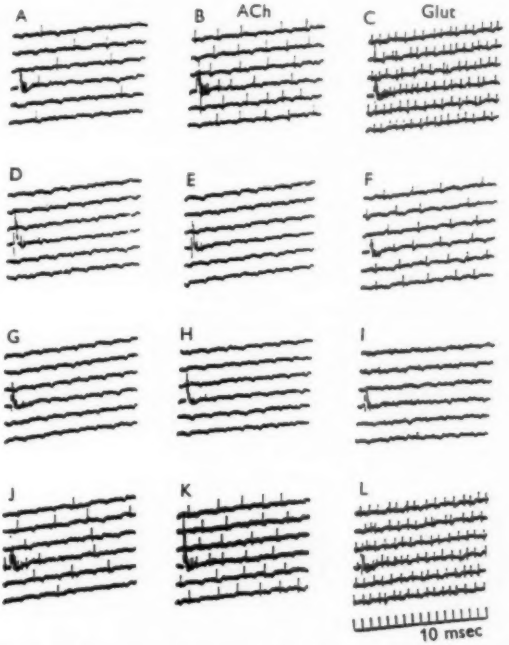


Fig. 5. Spike potentials recorded on moving film as in Fig. 1, from a single Renshaw cell and evoked by ventral-root stimulation (A, D, G and J), by iontophoretically applied acetylcholine (B, E, H and K) and by glutamate ions (C, F, I and L). A, B, C, control responses; D, E, F, 15, 7 and 12 sec respectively after a current of 100 nA began to pass atropine from one barrel of the five-barrel electrode; this current flowed for 18 sec; G, H and I, 5, 8 and 21 sec respectively after this current ceased; J, K, L, 3 min after the termination of the application of atropine. Time marker, 10 msec for all records.

Atropine

When administered intravenously, in comparatively large doses (2 mg/kg), atropine has but a slight depressant action upon the responses of Renshaw cells evoked by ventral root stimulation (Eccles, Fatt & Koketsu, 1954). A more powerful depression was observed when atropine was passed iontophoretically on to single Renshaw cells, the excitant action of iontophoretically applied acetylcholine also being blocked. In

addition, however, as with procaine, the repetitive activation of Renshaw cells by glutamate ions was blocked by atropine. This is illustrated in Fig. 5, which shows spike responses recorded from a Renshaw cell by means of the central barrel of a five-barrel electrode. The recording was made in a similar fashion to that of Fig. 1 and the control records A, B and C were evoked by a maximal ventral root volley, by acetylcholine and by glutamate ions respectively. In the latter two cases iontophoretic currents of 100 nA were used, this being cationic for acetylcholine and anionic for glutamate ions. An iontophoretic current of 100 nA then passed atropine from another barrel of the electrode for 18 sec, the responses D–F being recorded during this application, G–I shortly after the cessation of the iontophoretic current and J–L 3 min later, when complete recovery had occurred. The records D, G and J; E, H and K and F, I and L were evoked by the same stimuli as A, B and C respectively. This figure demonstrates that the synaptic activation of Renshaw cells as well as activation by acetylcholine and glutamate was reversibly suppressed by atropine, thus suggesting that this substance has a direct action upon the spike-potential generating mechanism. Alternatively, atropine could be blocking not only the cholinoceptive receptors but also those with which glutamic acid combines.

In view of the use of atropine in determining the cholinergic nature of transmission at junctional regions (Ambache, 1955) it was of interest to investigate if the cholinergic excitation of Renshaw cells was more susceptible to the action of this substance than that due to glutamate ion. The maximum frequencies at which applications of acetylcholine and glutamate ion fired a Renshaw cell are plotted in Fig. 6, with filled and open circles respectively. These substances were applied alternately at 8-sec intervals, with constant currents of 100 nA for periods of 4 sec. The magnitude of the current applying glutamate ion was chosen so that the frequency of firing was lower than that attained by acetylcholine. The constancy of the control responses to the left of the figure established that the interference between these applications was minimal. After the control responses a current of 15 nA passed atropine into the neighbourhood of the cell for 57 sec, and the alternating test applications of acetylcholine and glutamate continued during and after the passage of this drug. Throughout the series a ventral-root stimulus evoked responses from this cell and it was noted that when atropine blocked the responses to acetylcholine, these synaptically elicited spikes were reduced in number. However, there was very little alteration in the frequency of the firing produced by glutamate in this series, in spite of this substance being initially less effective in firing the cell than acetylcholine. With larger iontophoretic currents applying atropine, the spikes evoked by both glutamate and acetylcholine could be

blocked. Analyses such as these were performed upon several cells, and it was found that currents of from two to three times that necessary to block acetylcholine were necessary to suppress completely the excitation evoked by glutamate.

The responses of Fig. 7 show that atropine also suppressed the excitation of interneurones. These spike potentials were recorded extracellularly by means of the central barrel of a five-barrel electrode, those of Fig. 7A, B and C being evoked by a maximal dorsal root volley, whereas those of

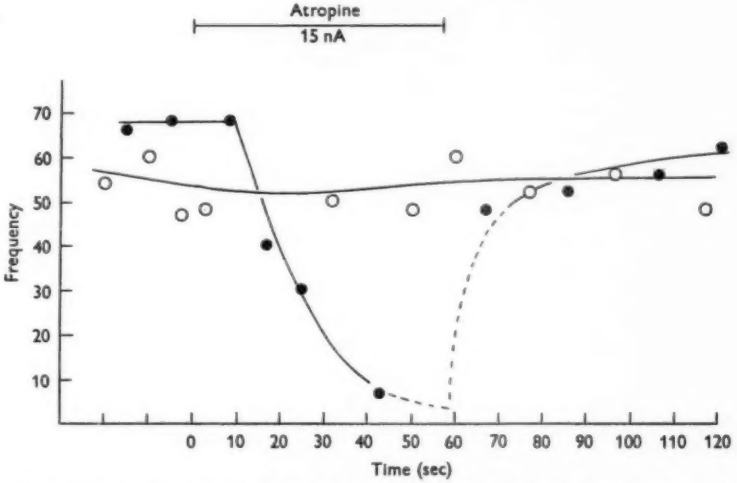


Fig. 6. Maximal frequencies of firing of a Renshaw cell evoked by applications of acetylcholine (●) and glutamate ions (○) passed iontophoretically from separate barrels of a five-barrel electrode, the central barrel of which was used for recording the responses. The applications were for periods of 4 sec, the substances being applied in turn approximately every 8 sec. During the time indicated by the horizontal bar, a current of 15 nA passed atropine from another barrel of the electrode. Ordinate, spikes per second.

Fig. 7D, E and F were produced by the iontophoretic application of glutamate ions. After the control responses A and D, a current of 250 nA was used to pass atropine out of the electrode for 30 sec and the traces of Fig. 7B, E and C, F were recorded 5 sec and 2 min after the cessation of this current, respectively. It is apparent that atropine depressed the production of both the synaptically and chemically evoked spikes of the interneurones. The observation that the excitant action of glutamate was blocked for some time after the cationic current applying atropine was terminated excludes the possibility that such action arose because of an interference between the currents applying these oppositely charged ions.

After applying atropine with currents of 100–200 nA for periods as long as 60 sec, the full recovery of both orthodromic and amino acid-evoked spikes of interneurones usually took 2–3 min. This action of atropine is very similar to that of procaine illustrated in Fig. 2. Although the iontophoretic method of applying drugs to neurones does not allow an accurate assessment to be made of relative potencies of action, the slower development of the blocking action of atropine with currents of 150–200 nA indicated that it was not as powerful a depressant as procaine.

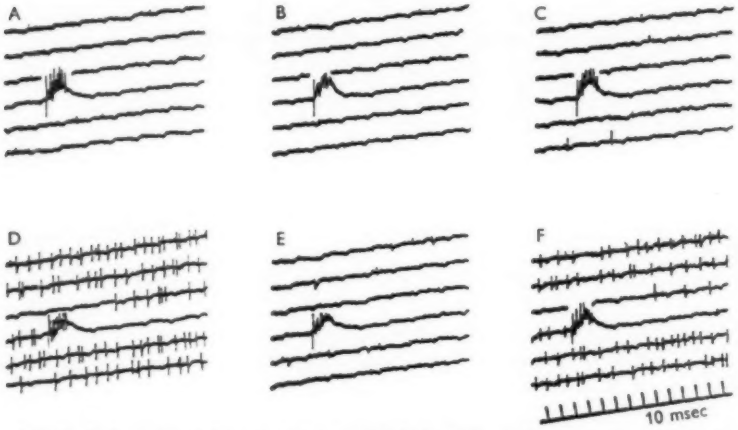


Fig. 7. Spike potentials recorded extracellularly from an interneurone of the L7 segment of the spinal cord by means of the central barrel of a five-barrel electrode. A, B and C, evoked by a dorsal root volley, this stimulus also being repeated in F, G and H; D, E and F, evoked by glutamate ion. In each case a current of 100 nA was used and the traces illustrated were recorded 3 sec after this current began. A, D, controls; B, E, 5 sec after the termination of a current of 250 nA passed atropine from the electrode; C, F, 2 min later than B, E; time marker, 10 msec.

In order to determine the action of atropine upon the membrane of motoneurones, it was applied extracellularly from the outer barrel of co-axial electrodes whilst intracellular records were obtained by means of the central barrel (cf. Fig. 3). The results were similar to those obtained with procaine, the records from one cell being illustrated in Fig. 8. This, a gastrocnemius motoneurone, had a resting potential of -50 to -58 mV for a period of 30 min and spike responses could be evoked by stimulation of the Group I gastrocnemius afferent fibres (A–F, (i)), the threshold of the spike being indicated by the horizontal arrow in each case. The volley arrival at the dorsal surface of the spinal cord was indicated by the triphasic potential of Fig. 8A–F, (ii). In addition, this cell could be excited

directly by a pulse of 0·3 msec duration which was passed through the intracellular electrode, as in Fig. 3. The size of this pulse (Fig. 8, A–F, (iii)) was varied in order to straddle the threshold of the cell, so producing spike potentials (Fig. 8A–F, (iv)) in approximately half of the six to eight tests. These tests were made 500 msec before the orthodromic responses, at 1 sec intervals.

In Fig. 8A (i) the spike potential was produced by a maximal Group I orthodromic volley, the threshold level of the EPSP for spike initiation being indicated by the horizontal arrow. Whilst the responses of Fig. 8B (i) were being recorded, the intensity of the stimulus applied to the gastrocnemius nerve was reduced in order to demonstrate the amplitude of the subthreshold, post-synaptic excitatory potentials; the threshold for excitation was about 3 mV. A current of 300 nA then passed atropine from the outer barrel of the co-axial electrode for 75 sec, the responses of C and D being recorded during this application and those of E and F after the termination of the current. Consideration of the orthodromic responses (i) shows that the threshold of the neuronal membrane was increased, the EPSP of Fig. 8D being 11 mV and yet failing to excite the cell, apart from one spike. After the current applying atropine ceased, the orthodromic activation of the cell recovered gradually, the threshold in Fig. 8F being approximately 3 mV. These observations were paralleled by the changes in the direct excitability of the membrane, a pulse of three times the size of the control (Fig. 8A) failing to evoke a spike in Fig. 8D. When connected to a direct-coupled amplifier, an intracellular electrode records not only the resting membrane potential of the impaled cell but also potentials due to the electrode itself. The 'resting' potential is determined as the difference between potentials recorded intracellularly and extracellularly, it being usual to assume that the 'electrode' potentials are identical under these circumstances. In many cells fluctuations are observed in the recorded potential which are not paralleled by changes in the excitability of the membrane. Consequently these changes in potential, of the order of 2–10 mV, are associated with the electrode. The potential of the cell from which the records of Fig. 8 were obtained had been steady during the 30 sec before the application of atropine. With the passage of iontophoretic current through the outer barrel of the co-axial electrode the recorded potential commenced to increase and continued to do so until at the cessation of current flow it was 8 mV greater than just before the beginning. The potential then remained constant at this new value for a further 140 sec. It might be considered that this 'hyperpolarization', seemingly associated with the action of atropine, accounts for the increase in threshold and decrease in membrane excitability of the cell. That such is not the case is demonstrated by the complete recovery of excitability

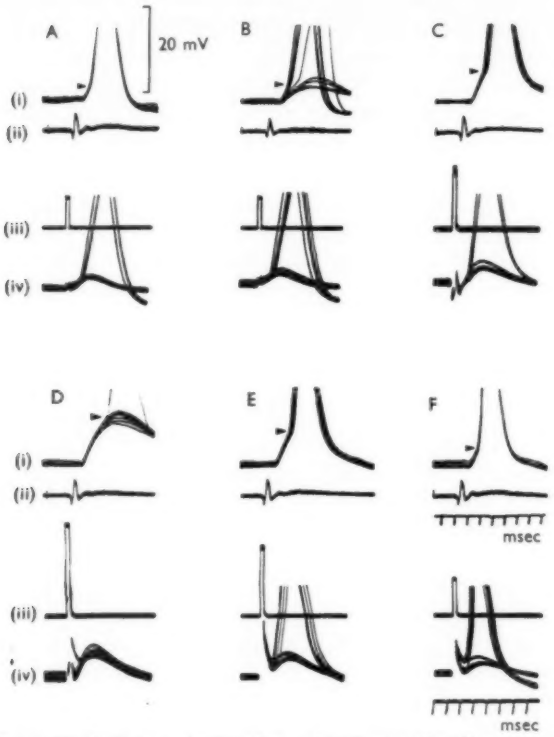


Fig. 8. Responses from a gastrocnemius motoneurone of resting potential −50 to −58 mV. All spike potentials are truncated and were recorded by the central barrel of a co-axial assembly.

(i) Orthodromic activation produced by stimulating the Group I afferent fibres of the gastrocnemius nerve, the arriving volley being recorded (ii) at the dorsal surface of the L7 segment of the cord. The threshold of the cell is marked by a horizontal arrow. The excitability of the cell was tested by means of a current pulse of 0·3 msec duration (iii) applied through the central barrel of the electrode. The size of this pulse was altered so that in approximately half the tests a spike potential was evoked (iv).

A, B, control responses, the size of the orthodromic volley being progressively decreased in B; C, D, 45 and 68 sec respectively after an iontophoretic current of 300 nA passed atropine from the outer barrel of the electrode: a current of 400 nA in the opposite direction had previously prevented this ion from leaving the electrode; E, F, 33 and 225 sec respectively after the atropine application was terminated. Each record consists of 4–8 responses at a frequency of 1/sec, the orthodromic and direct spikes being elicited 500 msec apart.

within 110 sec of the cessation of the current applying the atropine, in spite of the persisting 'hyperpolarization' (compare A, (i), (iii) and (iv) with F (i), (iii) and (iv)). The application of atropine to two other motoneurones with different co-axial electrodes resulted in similar changes of excitability without alteration in membrane potential. These results indicate, therefore, that atropine has a reversible depressant action upon the processes responsible for the rising phase of the spike potential of motoneurones.

DISCUSSION

Direct observations upon motoneurones of the lumbar segments of the cat indicate that procaine, in the absence of a change in membrane potential, raises the threshold of the neuronal membrane to direct, orthodromic and antidromic stimulation. There appears to be little or no change in either excitatory or inhibitory post-synaptic potentials or in the membrane resistance. Observations upon Renshaw cells and interneurones are in accordance with these findings, there being suppression of the spikes evoked either orthodromically or by excitant amino acids. In addition, it has been reported that the excitant action on neurones of compounds which chelate calcium ions is also prevented by procaine (Curtis, Perrin & Watkins, 1960). These results therefore indicate that procaine has a direct action upon the post-synaptic membrane of all of these types of neurone, independent of any effect it might have at the cholinoceptive receptors of Renshaw cells.

Thus, in common with the investigations upon squid axons (Shanes *et al.* 1959; Taylor, 1959), it can be concluded that procaine prevents the initiation of neuronal spikes, presumably by depressing the effectiveness with which depolarization of the membrane produces a high sodium permeability during the rising phase of the spike potential. Since the EPSP is unaltered and since there is probably an increased membrane permeability to sodium, as well as to other ions, during such a potential (Eccles, 1957), it can be concluded that the manner in which sodium ion passes through the membrane during the EPSP is different from that in which it does so during the spike. The progressive diminution in the size of the neuronal spikes produced by procaine can be accounted for by its blocking action upon areas of the neuronal membrane closest to the applying electrode, other portions of the cell continuing to be activated both chemically and synaptically. It is of interest that the membrane of motoneurones appears to be more susceptible to the action of procaine than that of the terminal afferent fibres having synaptic endings upon these cells. This conclusion is similar to that of Harmel & Malcolm (1958), who studied the action of procaine upon the motoneurones of the isolated frog's spinal cord.

Since extracellular records only were obtained from Renshaw cells, it could not be determined if procaine blocked the action of acetylcholine upon the post-synaptic receptors of these cells. However, since the end-plate potentials and potentials evoked by iontophoretic application of acetylcholine at the neuromuscular junction are reduced in size by this drug (del Castillo & Katz, 1957), it is very likely that procaine has a dual action upon Renshaw cells. Thus it is probable that procaine not only raises the threshold of the electrically excitable membrane of these cells, but also hinders the production of an EPSP by the synaptically released acetylcholine.

The results obtained in the present series of experiments indicate that atropine has actions similar to those of procaine, although it is probably not as effective a depressant of non-cholinoceptive neurones. The demonstration that this substance affects the electrical excitability of the motoneurone membrane renders unlikely the possibility that the blocking action of atropine upon the glutamate-induced spikes of Renshaw cells is due to this agent blocking the amino-acid receptor sites (p. 26). The actions of atropine upon cholinoceptive receptors and the sodium 'carrier' system are not surprising in view of the fact that both atropine and procaine are tertiary amino esters of aromatic acids and that atropine has approximately half the local anaesthetic potency of procaine (de Elio, 1948). In addition, many atropine analogues possess cocaine-like actions (Blicke & Kaplan, 1943). A variety of central actions of atropine have been described (Schweitzer & Wright, 1937; Bülbring & Burn, 1941; Calma & Wright, 1944; Feldberg, 1945; Rinaldi & Himwich, 1955; Bradley & Elkes, 1957; Feldberg, 1957) and many of these have been assumed to be due to the blocking action of this substance at cholinergic junctions, although Feldberg (1945) pointed out that atropine has a non-specific depressant action upon cells. The present results indicate that, since the concentration necessary to block the cholinergic excitation of Renshaw cells was lower than that which interferes with glutamate excitation by a factor of only two or three, care must be exercised when interpreting results obtained with this substance. Thus susceptibility to atropine may not of itself establish that synaptic transmission at a particular junctional region is cholinergic in nature.

Although direct evidence is lacking, atropine, being a tertiary amino compound, would be expected to penetrate the blood–brain barrier. Hence the relative ineffectiveness of this drug upon Renshaw cells following intravenous administration is presumably due to the lower local concentration attained by this method, compared with that obtained by iontophoretic application.

SUMMARY

1. Procaine and atropine have been applied iontophoretically to interneurones, motoneurones and Renshaw cells of the spinal cord of the cat, lightly anaesthetized with pentobarbital sodium.

2. The spike responses of such cells, whether elicited synaptically, antidromically, directly, or by the application of chemical excitants, were suppressed by these substances.

3. The post-synaptic and resting potentials of motoneurones were unaltered.

4. It is concluded that procaine and atropine, in addition to their blocking actions at cholinoceptive synapses, have a direct action on the post-synaptic membrane, presumably by preventing the activation of the sodium 'carrier'.

REFERENCES

AMBACHE, N. (1955). The use and limitations of atropine for pharmacological studies on autonomic effectors. *Pharmacol. Rev.* **7**, 467–494.

BLICKE, F. F. & KAPLAN, H. M. (1943). Synthetic mydriatics III. *J. Amer. chem. Soc.* **65**, 1967–1970.

BRADLEY, P. B. & ELKES, J. (1957). The distribution of cholinergic and non-cholinergic receptors in the brain: electrophysiological evidence. *Metabolism of the Nervous System*, ed. RICHTER, D., pp. 515–521. London: Pergamon Press.

BÜLBRING, E. & BURN, J. H. (1941). Observations bearing on synaptic transmission by acetylcholine in the spinal cord. *J. Physiol.* **100**, 337–368.

CALMA, I. & WRIGHT, S. (1944). Action of acetylcholine, atropine and eserine on the central nervous system of the decerebrate cat. *J. Physiol.* **103**, 93–102.

COOMBS, J. S., CURTIS, D. R. & ECCLES, J. C. (1957). The interpretation of spike potentials of motoneurones. *J. Physiol.* **139**, 198–231.

COOMBS, J. S., CURTIS, D. R. & ECCLES, J. C. (1959). The electrical constants of the motoneurone membrane. *J. Physiol.* **145**, 505–528.

COOMBS, J. S., ECCLES, J. C. & FATT, P. (1955). The electrical properties of the motoneurone membrane. *J. Physiol.* **130**, 291–325.

CURTIS, D. R. & ECCLES, R. M. (1958*a*). The excitation of Renshaw cells by pharmacological agents applied electrophoretically. *J. Physiol.* **141**, 435–445.

CURTIS, D. R. & ECCLES, R. M. (1958*b*). The effect of diffusional barriers upon the pharmacology of cells within the central nervous system. *J. Physiol.* **141**, 446–463.

CURTIS, D. R., PERRIN, D. D. & WATKINS, J. C. (1960). The excitation of spinal neurones by the ionophoretic application of agents which chelate calcium. *J. Neurochem.* (in the Press).

CURTIS, D. R., PHILLIS, J. W. & WATKINS, J. C. (1959). The depression of spinal neurones by γ-amino-*n*-butyric acid and β-alanine. *J. Physiol.* **146**, 185–203.

CURTIS, D. R., PHILLIS, J. W. & WATKINS, J. C. (1960). The chemical excitation of spinal neurones by certain acidic amino acids. *J. Physiol.* **150**, 656–682.

DE ELÍO, F. J. (1948). Acetylcholine antagonists: a comparison of their action in different tissues. *Brit. J. Pharmacol.* **3**, 108–112.

DEL CASTILLO, J. & KATZ, B. (1957). A study of curare action with an electrical micromethod. *Proc. Roy. Soc.* B, **146**, 339–356.

ECCLES, J. C. (1957). *The Physiology of Nerve Cells.* Baltimore: Johns Hopkins Press.

ECCLES, J. C., FATT, P. & KOKETSU, K. (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. *J. Physiol.* **126**, 524–562.

FELDBERG, W. (1945). Present views on the mode of action of acetylcholine in the central nervous system. *Physiol. Rev.* **25**, 596–642.

FELDBERG, W. (1957). Acetylcholine. *Metabolism of the Nervous System*, ed. RICHTER, D., pp. 493–509. London: Pergamon Press.

FURUKAWA, T. (1957). Properties of the procaine end-plate potential. *Jap. J. Physiol.* **7**, 199–212.

HARMEL, M. & MALCOLM, J. L. (1958). The site of action of procaine on the isolated spinal cord of the frog. *J. Physiol.* **140**, 213–219.

HARVEY, A. M. (1939). The actions of procaine on neuromuscular transmission. *Johns Hopk. Hosp. Bull.* **65**, 223–238.

RINALDI, F. & HIMWICH, H. E. (1955). Alerting responses and actions of atropine and cholinergic drugs. *Arch. Neurol. Psychiat., Chicago*, **73**, 387–395.

SCHWEITZER, A. & WRIGHT, S. (1937). The action of eserine and related compounds and of acetylcholine on the central nervous system. *J. Physiol.* **89**, 165–197.

SHANES, A. M., FREYGANG, W. H., GRUNDFEST, H. & AMATNIEK, E. (1959). Anaesthetic and calcium action in the voltage clamped squid giant axon. *J. gen. Physiol.* **42**, 793–802.

TAYLOR, R. E. (1959). Effect of procaine on electrical properties of squid axon membrane. *Amer. J. Physiol.* **196**, 1071–1078.

J. Physiol. (1960), **153**, *pp.* 35–51
With 10 *text-figures*
Printed in Great Britain

SCRATCHING MOVEMENTS AND FACILITATION OF THE SCRATCH REFLEX PRODUCED BY TUBOCURARINE IN CATS

BY F. R. DOMER* AND W. FELDBERG
From the National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 22 *February* 1960)

Recently Feldberg & Fleischhauer (1960) have shown that in cats anaesthetized with pentobarbitone sodium, tubocurarine applied to the dorsal surface of the exposed cervical cord at the level of C1 produces muscular activity in the hind legs. As the pattern of this activity resembled that of the scratch reflex, they concluded that tubocurarine elicits scratching movements by its action on a restricted area of the upper cervical cord. The present experiments are a continuation of this work. They show that tubocurarine is effective also when applied to the level of C2 and that besides eliciting scratching movements, it facilitates the scratch reflex which can be evoked by rubbing the pinna of the ear or the skin behind the ear. When the effects of tubocurarine were compared in cats anaesthetized with pentobarbitone sodium and in decerebrate cats, a difference in the pattern of movements was observed. The movements were usually more rhythmic during pentobarbitone sodium anaesthesia and more sustained in decerebrate cats.

METHODS

Cats weighing 2·1–3·5 kg were used. They were either anaesthetized with pentobarbitone sodium (33 mg/kg) injected intraperitoneally, additional amounts being injected during the course of prolonged experiments, or decerebrated at the anterior collicular level under ethyl chloride–ether anaesthesia.

The experimental procedures were essentially those employed by Feldberg & Fleischhauer (1960). The cat was lying on its belly and the head was fixed to the ear bars of a head holder, similar to the Horsley–Clark stereotaxic instrument. The upper cervical cord was exposed and myographic records were taken from the tibialis anterior muscles.

In order to apply tubocurarine to the exposed dorsal surface of the cervical cord, tubocurarine chloride was dissolved in an artificial cerebrospinal fluid (c.s.f.) of the composition given by Merlis (1940). A pledget of cotton wool was soaked in this solution and placed on the desired region of the cervical cord. The procedure frequently adopted was that of leaving the tubocurarine on the cord for periods of 15 min and washing the cord with pledgets soaked in artificial c.s.f. for periods of 30 min. The pledgets were changed every few minutes.

* Life Insurance Medical Research Fund Fellow.

The scratch reflex was evoked by rubbing either the pinna of the ear between the fingers or the skin of the neck behind the ear.

In two experiments the effect of chloralose was investigated during pentobarbitone sodium anaesthesia. The chloralose (30 mg/kg) was injected, in a 1% solution, into the superficial vein of the right foreleg.

In several experiments the medulla oblongata was transected below the obex, sometimes after hemisection at the level indicated in Fig. 1 of Feldberg & Fleischhauer (1960).

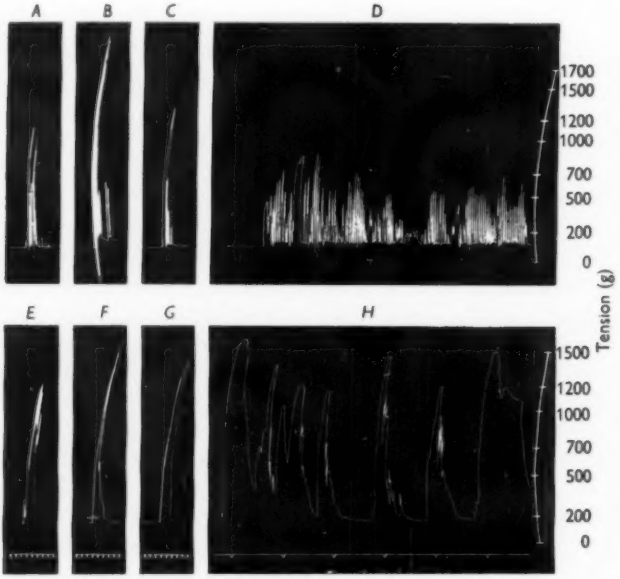


Fig. 1. Records of anterior tibialis muscles of four cats anaesthetized with pentobarbitone sodium (*A* to *D*), and of four decerebrate cats (*E* to *H*). Muscular activity produced by application of tubocurarine 1/1000 to the dorsal surface of the cervical cord at the level of C1, with the exception of cat G where it was applied at the level of C2. Time marker, 10 sec.

RESULTS

Cats anaesthetized with pentobarbitone sodium

Scratching movements of the hind legs were obtained not only on application of tubocurarine dissolved in artificial c.s.f. to the dorsal surface of the cervical cord at the level of C1, but also down the cord to the level of C2, although here higher concentrations of the drug were required.

Spontaneous muscular activity. Figure 1 shows typical bursts of scratching movements at *A–C* and a period of more continuous rhythmic activity at *D* during application of tubocurarine 1/1000 at the level of C1. The effects, which are often associated with extension and adduction of the ipsilateral

foreleg, are similar to those described by Feldberg & Fleischhauer (1960). In one experiment the muscular activity in the hind leg consisted of intermittent sustained contractions. The onset of this activity in the left tibialis muscle is illustrated at *H* in Fig. 2.

When applied at the level of C1, tubocurarine in a concentration of 1/2000 was effective in all experiments; a concentration of 1/5000 was tested in two experiments but produced muscular movements in only one.

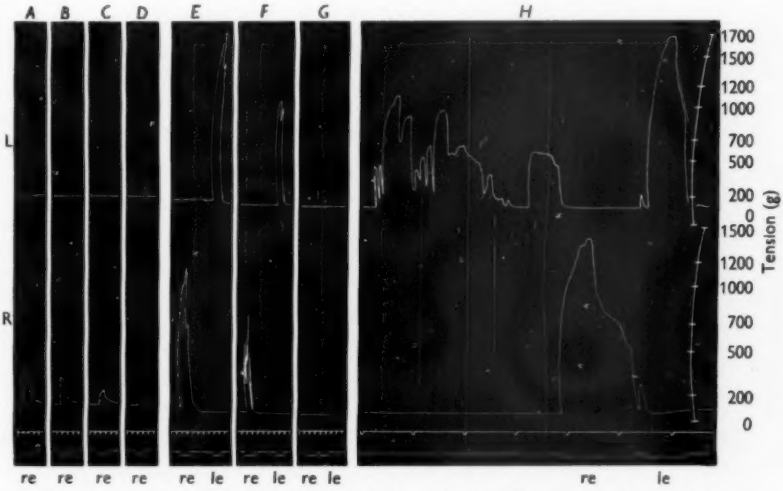


Fig. 2. Records of left (L) and right (R) anterior tibialis muscles of a cat anaesthetized with pentobarbitone sodium. *A*, a few minutes after exposure of the upper cervical cord before application of tubocurarine; *B*, *C* and *D*, 5, 10 and 15 min later. *E*, during application of tubocurarine 1/5000 to the dorsal surface of the cervical cord at the level of C1. *F* and *G*, 10 and 20 min after removal of the tubocurarine. *H*, during application of tubocurarine 1/2000 at the level of C1. On the right, tension in grams. Bottom signals indicate the periods of rubbing the left or right ear (le and re). Time marker, 10 sec.

In this experiment 1/10,000, applied for 15 min, was weakly effective. The response was delayed until 4 min after removal of the tubocurarine and consisted of a few contractions in one leg only. The tension developed in the tibialis muscle was approximately 100 g.

When the tubocurarine was removed and the surface of the cervical cord was washed repeatedly with pledgets soaked in artificial c.s.f., the muscular activity gradually subsided, the contractions becoming progressively weaker and less frequent, and ceasing within 15–40 min, according to the concentration of the tubocurarine used and the duration of its application.

Tubocurarine applied at the level of C2 in a concentration of 1/2000 produced no muscular movements; 1/1000 did so, but not in all experiments, and sometimes in one hind leg only, although the tubocurarine was applied to both sides of the cervical cord. In some experiments the effect consisted of a few weak contractions which occurred after a long delay; in others the contractions were as strong as those obtained with tubocurarine

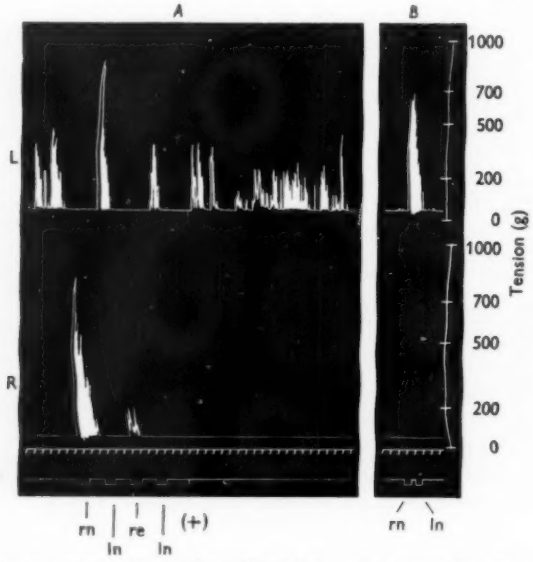


Fig. 3. Records of left (L) and right (R) tibialis anterior muscles of a cat anaesthetized with pentobarbitone sodium. *A*, during the end of a 15 min period of application of tubocurarine 1/1000 to the dorsal spinal surface at the level of C2. Tubocurarine removed at (+). *B*, 25 min after removal of the tubocurarine. Bottom signal indicates the periods of rubbing the left or right ear (le and re) and the skin of the neck behind the left or right ear (ln and rn); for details see text. Time marker, 10 sec.

1/1000 applied at the level of C1. Figure 3 illustrates an experiment in which the application of tubocurarine 1/1000 at the level of C2 caused contractions in the left hind leg only.

In one experiment tubocurarine was applied transversely to a restricted region between the levels of insertion of the sensory roots of C1 and C2, by means of a cotton thread used as a wick. Spontaneous scratching movements occurred, as illustrated in Fig. 4.

Tubocurarine 1/1000 applied at the level of C3 failed to produce muscular effects, even in those experiments in which the application at the level of C2 caused strong contractions.

Scratching movements elicited by skin stimulation. Before the tubocurarine was applied, rubbing the pinna of the ear or the skin behind the ear usually produced no muscular effects. In a few experiments, however, there was a short period immediately following the exposure of the upper cervical cord during which such stimulation produced scratching movements in the ipsilateral hind leg. This over-excitability always decreased rapidly and the skin stimulation became ineffective within 15–30 min. In one experiment the response to rubbing an ear during this initial period of

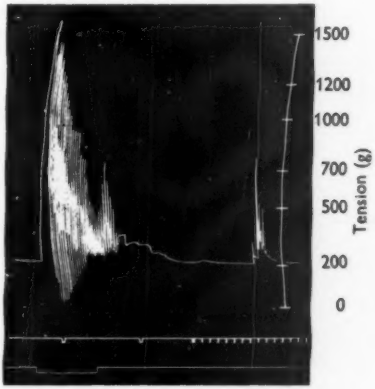


Fig. 4. Record from the left tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. A scratch reflex evoked on rubbing the pinna of the left ear and a spontaneous burst of smaller scratching movements during application of tubocurarine 1/1000 between the levels of insertion of the sensory roots of C1 and C2 by means of a cotton thread used as a wick. Bottom signal indicates the period of rubbing the pinna of the left ear. Time marker, 10 sec.

over-excitability did not consist of rhythmic beats but of a weak sustained contraction, as illustrated at *A*, *B* and *C* in Fig. 2. This effect, which occurred on the left side only, was obtained in the experiment in which the spontaneous movements on application of tubocurarine also consisted of sustained contractions.

When tubocurarine was applied to the region of C1 in a concentration evoking spontaneous movements, bursts of scratching movements could always be elicited in a hind leg on rubbing the ipsilateral ear, but not on rubbing the skin behind the ear. When a scratching response was evoked by rubbing the ear, any spontaneous activity present in the opposite hind leg ceased. Rubbing the ear did not evoke movements in the foreleg. A typical evoked scratching response of the left tibialis muscle is illustrated in Fig. 5. The response was obtained on the application of tubocurarine 1/2000 during an interval between spontaneous movements, and was

recorded on a fast drum the better to illustrate the individual beats. The left ear was rubbed for about 7 sec; the response began as soon as the ear was touched, but rhythmic beats continued for a few seconds after cessation of stimulation. The evoked scratching movements were often stronger than those occurring spontaneously (see Figs. 3 and 6).

In the one experiment in which the spontaneous movements following application of tubocurarine 1/1000 at the level of C1 consisted of sustained irregular contractions, rubbing the ears also produced sustained contractions without rhythmic beats, as is shown at *H* in Fig. 2.

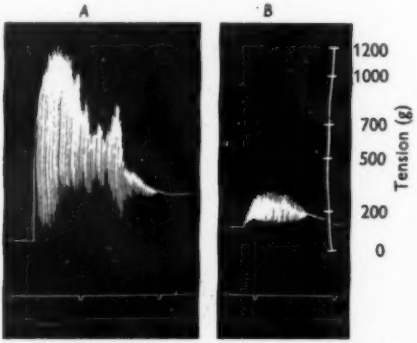


Fig. 5. Records from the left tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. Two scratching responses evoked on rubbing the pinna of the left ear during application of tubocurarine 1/2000 (at *A*) and 1/1000 (at *B*) to the dorsal surface of the cervical cord at the level of C1. *B* was taken a few minutes after transection of the medulla just below the obex. Bottom signals indicate the periods of rubbing the pinna. Time marker, 10 sec.

In those experiments in which tubocurarine 1/1000 applied to the region of C2 produced spontaneous movements, scratching movements could be evoked by rubbing the pinna and sometimes also by rubbing the skin behind the ear. In fact, in one experiment this stimulus evoked stronger scratching movements than rubbing the pinna. Usually, however, rubbing the pinna was the stronger, and sometimes the only effective, stimulus. Figure 3 illustrates an experiment in which the scratching movements evoked from the pinnae were stronger than those evoked by rubbing the skin behind the ears; they were also stronger than the movements which occurred spontaneously.

In the one experiment in which the tubocurarine was applied transversely to a restricted region between the levels of insertion of the sensory roots of C1 and C2, rubbing the pinna evoked a strong scratch reflex (see Fig. 4).

The scratching movements evoked on skin stimulation occurred not only

during periods of spontaneous movements caused by the application of tubocurarine, but also during the latent period before the spontaneous movements began and for some time after their cessation on removing the tubocurarine from the cervical cord. A concentration of tubocurarine too weak to produce spontaneous movements was sufficient to evoke scratching movements on skin stimulation. Some of these results are seen in Figs. 2 and 3.

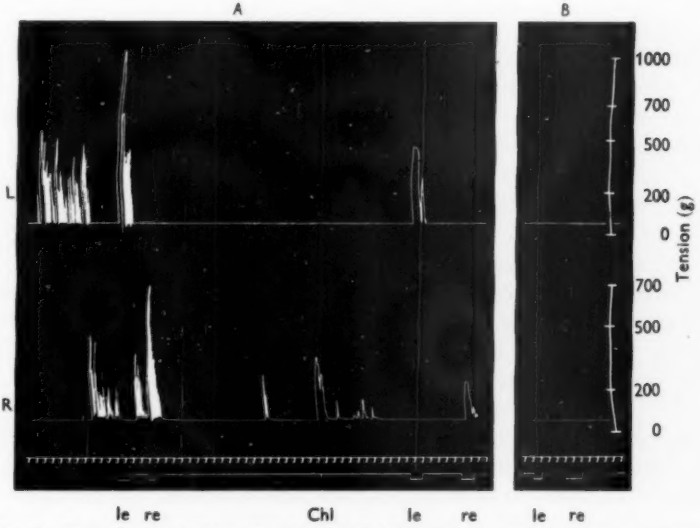


Fig. 6. Records of left (L) and right (R) tibialis anterior muscles of a cat anaesthetized with pentobarbitone sodium. Tubocurarine 1/1000 had been applied for 15 min to the dorsal surface of the cervical cord at the level of C1. *A*, about 15 min after removal of the tubocurarine. *B*, 20 min after *A*, during renewed application of tubocurarine. Bottom signals indicate the periods of rubbing the pinna of the left or right ear (le and re) and the intravenous injection of chloralose 30 mg/kg (Chl). Time marker, 10 sec.

In the experiment of Fig. 2, tubocurarine 1/5000 applied at the level of C1 for 15 min produced no muscular activity, yet during and for some time after its application rubbing a pinna evoked strong responses. These consisted of a sustained contraction without rhythmic beats (at *E* and *F*). In this experiment the spontaneous muscular movements were also sustained contractions.

In the experiment of Fig. 3 (at *A*), the application of tubocurarine 1/1000 to the level of C2 produced spontaneous movements in the left hind leg only, yet rubbing the ear or the skin behind it evoked ipsilateral scratching responses in both hind legs. Thirty minutes after removal of the

tubocurarine, when the spontaneous activity in the left tibialis muscle had ceased, rubbing the left ear still produced the characteristic scratching response (Fig. 3 at *B*).

As the evoked scratching responses gradually subsided after removal of the tubocurarine, they not only became weaker but were often delayed as well. The movements no longer began immediately on touching the sensitive skin areas, but only after they had been rubbed for several seconds.

Apart from the two restricted areas, rubbing the skin of any other part of the body, such as the shoulder, the ribs, or the face, did not evoke scratching movements when tubocurarine was applied to the level of C1 or C2. Touching the whiskers was also ineffective.

When tubocurarine 1/1000 was applied to the level of C3, rubbing the skin of any part of the body was ineffective in evoking scratching movements.

Additional pentobarbitone sodium and chloralose. When a dose of pentobarbitone sodium 15 mg/kg, which is a little less than half the dose used initially for the anaesthesia, was injected intraperitoneally, the spontaneous activity produced by the tubocurarine was attenuated for 2–2½ hr. The strength of contractions of the tibialis muscles diminished and the intervals between the bursts of activity lengthened. The degree of attenuation varied in different experiments.

In two cats anaesthetized with pentobarbitone sodium chloralose 30 mg/kg was injected intravenously. This dose, which is a little less than half the full anaesthetic dose, abolished the muscular activity produced by tubocurarine, as is shown in Fig. 6. Both the spontaneous activity and the evoked responses were abolished, without recovery during the following 3 hr.

Hemisection and transection of the medulla. Hemisection of the medulla below the obex did not affect, or only slightly decreased the strength of contractions produced by tubocurarine or of evoked responses in both hind legs. Transection of the medulla, whether or not preceded by hemisection, always resulted in an immediate large reduction in the strength of the spontaneous movements. The evoked responses were also greatly reduced but, as is illustrated in Fig. 5*B*, their rhythm remained unchanged. In those experiments which were continued for 1½–2 hr after the transection the reduced activity persisted.

Decerebrate cats

Feldberg & Fleischhauer (1960) showed that decerebration did not abolish the spontaneous movements which occur on topical application of tubocurarine to the upper cervical cord. In their experiments decerebration was carried out during pentobarbitone sodium anaesthesia, so that

the effect of this anaesthetic was not excluded. In the present experiments decerebration was performed under a volatile anaesthetic and the tubocurarine effect was investigated later, at a time when it could no longer be influenced by the anaesthetic agent. The characteristics of the response to tubocurarine in these animals were that the strength of the movements in the hind legs was usually stronger, and that the movements tended to be more sustained than in cats anaesthetized with pentobarbitone sodium. These differences applied to the spontaneous activity and to the evoked responses. Movements of the foreleg were also much more pronounced.

Spontaneous muscular activity. When tubocurarine was applied at the level of C1 or C2, spontaneous movements in the hind legs began after a latency which varied between 2 and 15 min. The flexion of the hind leg which initiates a scratching movement was often sustained and not followed by the typical strong beats, so that the response was not easily recognizable as a scratching movement. The tension developed in the tibialis muscles sometimes reached 2 kg or more. Relaxation occurred with or without rhythmic beats. Succeeding similar contractions often followed at random. Sometimes the activity in the tibialis muscle was interrupted by periods of quiescence lasting several minutes. When the tubocurarine was applied by means of a cotton thread to one side of the cord, the response occurred in the ipsilateral leg; when applied to both sides, responses occurred in both hind legs, but alternately. When the application was continued the maximum tension developed during these contractions tended to decrease. On removal of the tubocurarine from the spinal cord the subsidence of spontaneous movements was the same as that described in cats anaesthetized with pentobarbitone sodium. Figure 1 illustrates, in the lower records, typical sustained contractions of the tibialis muscle with no, or only a few, signs of rhythmic beats and, in contrast, in the upper records, the rhythmic pattern of movements obtained under pentobarbitone sodium anaesthesia. The contractions at *E*, *F* and *G* last approximately 20 sec. They are followed by periods of quiescence and correspond to the bursts of rhythmic beats seen during pentobarbitone anaesthesia. At *E* such rhythmic beats are in fact seen to be superimposed on the strong contraction. Even when the record suggests a single sustained contraction, there are often small rhythmic movements of the foot which are too weak to be recorded by the lever system used. At *H* the record is taken on a faster drum and illustrates the random nature of the sustained contractions.

In a few experiments the muscular effect consisted of typical scratching movements or bursts of rhythmic beats characteristic of the experiments in pentobarbitone sodium anaesthesia. In yet other experiments the muscular effect began with a burst of rapid rhythmic activity, followed

by more sustained irregular contractions. In all experiments in which the pattern of movements consisted mainly of irregular sustained contractions there were, nevertheless, from time to time, periods in which bursts of rhythmic beats occurred.

The pattern and strength of muscular movements were essentially the same whether the tubocurarine was applied at the level of C1 or C2, but at the level of C1 lower concentrations were effective. At C1 spontaneous movements occurred with a concentration as low as 1/5000, but at C2 only with 1/1000, and at C3 even 1/1000 was ineffective.

Strong activity in a hind leg was associated with extension, elevation and adduction in the ipsilateral foreleg, whilst the claws were often protruded. The movement resembles that made by a cat when wiping something off its face.

Scratching movements elicited by skin stimulation. Before the application of tubocurarine, scratching movements on rubbing the ear, or the skin behind it, were obtained only immediately following the exposure of the dorsal surface of the upper cervical cord. The over-excitability, as in cats under pentobarbitone sodium, decreased rapidly and the skin stimulation became ineffective.

When tubocurarine was applied at the level of C1, rubbing the ear evoked scratching movements in the ipsilateral hind leg, often accompanied by movements of the ipsilateral foreleg. Rubbing the skin behind the ear was ineffective. When tubocurarine was applied at the level of C2, scratching movements of the hind leg were regularly obtained on rubbing the skin behind the ear, but seldom on rubbing the pinna itself. With both sites of application rubbing the skin of any other part of the body was ineffective. When tubocurarine was applied to one side of the cervical cord, the evoked scratching movements were obtainable on the same side only. When tubocurarine was applied at the level of C3, no scratching movement could be evoked on rubbing the ear, the skin behind it, or elsewhere.

The scratching movements evoked by skin stimulation were obtained in the period preceding spontaneous movements, during spontaneous activity, and for some time after it had ceased following removal of the tubocurarine. When spontaneous activity was present the evoked movements were most clearly demonstrated during intervals of quiescence. They could also be elicited with tubocurarine in concentrations too weak to produce spontaneous movements, for instance, with 1/10,000 applied at the level of C1 and with 1/2000 at the level of C2.

The pattern of the evoked scratching movement was usually a sustained contraction with or without a superimposed series of weak beats. Sometimes, however, it consisted of a series of strong rhythmic beats. This appeared to be related to whether the spontaneous activity consisted

primarily of bursts of rhythmic beats, or, as was more usual, of sustained contractions; in general, however, the evoked responses tended to be more rhythmic than the spontaneous movements. The evoked responses sometimes outlasted the period of skin stimulation for a few seconds and were often stronger than those occurring spontaneously. Representative results are illustrated in Figs. 7, 8 and 9. In the experiment of Fig. 7 tubocurarine 1/1000 was applied for 15 min to both sides of the cord at the

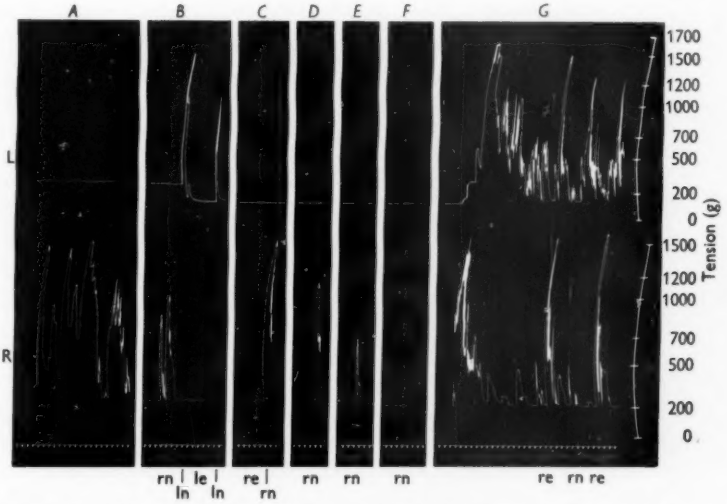


Fig. 7. Records of left (L) and right (R) anterior tibialis muscles of a decerebrate cat. *A*, during application of tubocurarine 1/1000 dorsally at the level of C2. *B*, 8 min after removal of the tubocurarine and subsidence of the spontaneous movements. *C*, *D*, *E* and *F*, 15, 30, 35 and 40 min, respectively, after removal of the tubocurarine. *G*, during application of tubocurarine 1/1000 at the level of C1. Bottom signal indicates the periods of rubbing the pinna of the left or right ear (le and re) and the skin of the neck behind the left or right ear (ln and rn); for details see text. Time marker, 10 sec.

level of C2. Spontaneous muscular activity occurred on the right side only (at *A*); yet (at *B*) the right hind leg as well as the left responded to stimulation of the skin behind the ear, but rubbing the pinna itself was ineffective. Following removal of the tubocurarine this evoked response disappeared on the left side within 15 min whilst it was still obtainable on the right side (at *C*). It decreased gradually on this side too, and 35 min after removal of the tubocurarine the response was reduced and occurred only after the skin had been rubbed for 20 sec (at *E*). Forty minutes after removal of the tubocurarine the response was absent (at *F*). On the other

hand, when tubocurarine was applied at the level of C1, rubbing the ears produced strong evoked responses in both legs. This is illustrated for the right tibialis muscle at *G*. The moment the ear was touched a strong contraction occurred followed by weaker rhythmic beats. Rubbing the skin behind the ear was ineffective.

Figures 8 and 9 illustrate two patterns of responses evoked by rubbing the ear, recorded on a faster drum. The effects were obtained after the

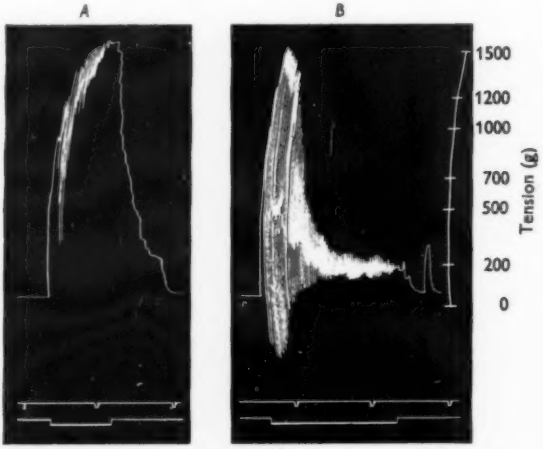


Fig. 8. Records of the left anterior tibialis muscle of a decerebrate cat. Evoked responses on rubbing the pinna of the left ear before (at *A*) and 2 hr after (at *B*) an intraperitoneal injection of pentobarbitone sodium 30 mg/kg. The responses were obtained a few minutes after removal of tubocurarine 1/5000 (at *A*) and 1/1000 (at *B*), after spontaneous activity had ceased. The tubocurarine had been applied dorsally at the level of C1 each time for 15 min. Bottom signals indicate the periods of rubbing the pinna of the left ear; for details see text. Time marker, 10 sec.

disappearance of spontaneous movements following removal of tubocurarine from the region of C1. In the experiment of Fig. 8 the pattern of spontaneous activity produced by tubocurarine had been that of sustained irregular contractions, whereas Fig. 9 is from one of the few experiments in which the spontaneous activity was characterized by bursts of rhythmic beats. These two patterns are reflected in the evoked responses as well. Figure 9 also illustrates that the evoked response occurs in the ipsilateral leg only.

Effect of pentobarbitone sodium. In the decerebrate cat the intraperitoneal injection of pentobarbitone sodium 20–30 mg/kg not only diminished the strength of the spontaneous contractions to a varying extent but also changed their character, i.e. they became more rhythmic. The evoked

responses showed the same change but did not necessarily decrease in strength. These results are illustrated in Figs. 8 and 10. Figure 8 shows the change in pattern of the evoked response on rubbing the left ear and Fig. 10, from the same experiment, the changes in spontaneous activity

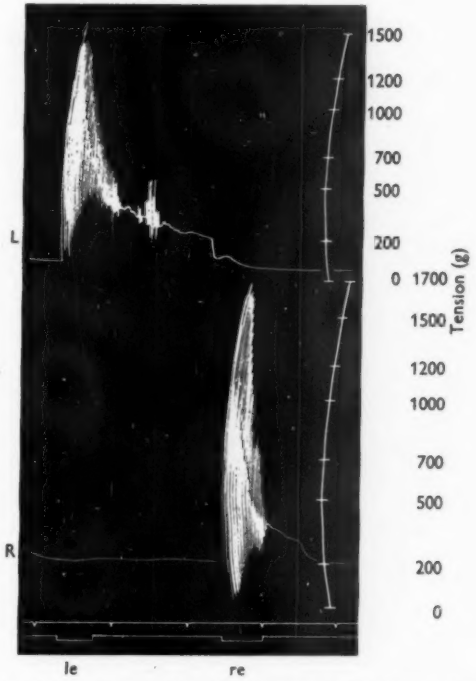


Fig. 9. Records of the left (L) and right (R) anterior tibialis muscles of a decerebrated cat. Evoked responses on rubbing the left (le) and right (re) ear a few minutes after removal of tubocurarine 1/1000 and after cessation of spontaneous activity. The tubocurarine had been applied dorsally at the level of C1 for 15 min. Bottom signals indicate the periods of rubbing the pinnae of the ears. Time marker, 10 sec.

following intraperitoneal injection of pentobarbitone sodium 30 mg/kg. The spontaneous activity produced by the application of tubocurarine 1/5000 decreased in the left hind leg and became more rhythmic in character. In the right hind leg it was abolished, but rubbing the ear was still effective (at *B*). When the concentration of tubocurarine was increased to 1/1000 spontaneous activity reappeared in both legs (at *C*).

Transection of the medulla. Transection of the medulla below the obex greatly reduced spontaneous activity produced by tubocurarine as well as

the evoked responses, which in addition became more rhythmic in character. Unlike the results obtained in the cats under pentobarbitone sodium anaesthesia, in decerebrate cats the activity ceased within 10 min of transection.

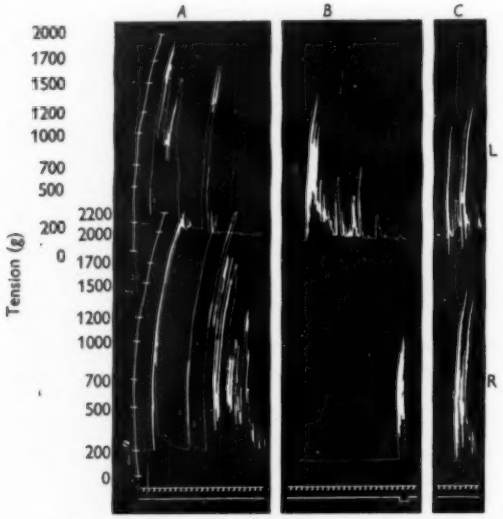


Fig. 10. Records of left (L) and right (R) anterior tibialis muscles of a decerebrate cat before (at *A*) and after (at *B* and *C*) intraperitoneal injection of pentobarbitone sodium 30 mg/kg, during the dorsal application of tubocurarine 1/5000 (at *A* and *B*) and 1/1000 (at *C*) at the level of C1. Bottom signal in *B* indicates a period of rubbing the pinna of the right ear. Time marker, 10 sec.

DISCUSSION

Feldberg & Fleischhauer's (1960) conclusion that the movements seen during tubocurarine application to a restricted area of the cervical cord at the level of C1 were scratching movements was based on the observed similarity between these movements and those a cat makes when scratching itself. The movements of the hind legs were considered to be those of a scratch reflex. These conclusions are no longer based solely on this similarity but also on the finding that tubocurarine facilitates the scratch reflex evoked on rubbing the pinna of the ear or the skin behind it.

In the present experiments scratching movements as well as facilitation of the scratch reflex were obtained when the tubocurarine was applied not only at the level of C1 but also at the level of C2. At the level of C2, however, a stronger concentration of tubocurarine was required to produce these effects; even a concentration of 1/1000 was not always effective. This

explains why Feldberg & Fleischhauer did not obtain an effect when they applied the tubocurarine at this level. The present results are in accord with the assumption that the tubocurarine acts at the synapses of the afferent filaments of those nerve roots which are activated in the scratch reflex. It is known from the early experiments of Sherrington (1908–9, 1910, 1917) on the scratch reflex in cats that these roots are those of C1 and C2 and of the 5th cranial nerve, all of which have synapses at the spinal levels where the tubocurarine was applied. Sherrington had found that stimulation of these roots elicits the scratch reflex, as did stimulation of cross-sections of the bulb or cord at points in the little crescentic field formed by the descending root of the trigeminal nerve which extends to the level of C2 (Gerard, 1923) and is undoubtedly reached by the tubocurarine when applied at this level. The evidence for this is the finding of Feldberg & Fleischhauer (1960) that the dye bromophenol blue which, like tubocurarine, produces scratching movements, stains this field intensely when applied to the dorsal surface of the cervical cord at the level of C1.

Sherrington (1910) found that in cats the scratch reflex is also elicited from skin regions, the afferent fibres of which reach the cord at a lower level than C2, i.e. 'from the skin covering the shoulder, the back and outer edge of the upper arm even down to the elbow'; yet when applied at the level of C3 the tubocurarine was ineffective. This could be due to the fact that in this region the synapses of the afferent filaments are located more remotely from the surface or that they are less abundant. The latter possibility is compatible with Sherrington's (1910) finding that 'the skin about the root of the pinna and just behind it...appears to constitute the region whence the scratch reflex...is most easily excited'.

Sherrington (1908–9) regularly obtained the scratch reflex on rubbing the pinna or the neck behind it in normal, decapitate and decerebrate cats. In the present experiments these stimuli were ineffective in eliciting the scratch reflex before the tubocurarine application. Only occasionally did they elicit a scratch reflex and then only during a short period following the exposure of the upper cervical cord. This disparity can be accounted for by differences in the experimental set-up. In our experiments the head of the cat was immobilized with the aid of ear bars which produced a constant stimulus. It is possible that this stimulus suppressed the scratch reflex evoked on rubbing the ear or the skin behind it, either because this stimulus is capable of causing the scratch reflex and adaptation has occurred, or because the stimulus is of a kind which inhibits the normal scratch reflex. Therefore the conditions in our experiments required the facilitation by tubocurarine in order to elicit the scratch reflex.

As synapses of the filaments of the trigeminal nerve extend down to the level of C2, it is not possible to say whether the site of action of

tubocurarine is mainly on these synapses or on those formed by the sensory filaments of C1 and C2. It is interesting, however, that stimulation by tubocurarine of the restricted area between the sensory filaments of C1 and C2 is sufficient to cause both spontaneous scratching movements and facilitation of the scratch reflex.

The present experiments confirm the observation of Feldberg & Fleischhauer (1960) that chloralose abolishes the effect of tubocurarine. This effect was obtained with less than half the dose normally used for full anaesthesia. Pentobarbitone sodium also affected the tubocurarine responses but usually did not abolish them. The responses were diminished in strength. When the effect of pentobarbitone sodium was investigated in decerebrate cats the responses became not only attenuated but often also more rhythmic in character. The attenuating effect of pentobarbitone sodium is compatible with the observation that it reduces the convulsive activity which is elicited in unanaesthetized cats by an injection of tubocurarine into the cerebral ventricles (Feldberg & Sherwood, 1954). No explanation is apparent for the observation that when tubocurarine was applied to the level of C2 in decerebrate cats a scratch reflex was usually elicited more easily by rubbing the skin behind the ear than by rubbing the pinna itself, whereas in cats anaesthetized with pentobarbitone sodium rubbing the pinna was the more effective stimulus.

The finding that transection of the medulla greatly reduces, but does not abolish, the effects of tubocurarine suggests that the higher nervous centres enhance the tubocurarine effects, but that the pattern of the scratch reflex and of the scratching movements is intrinsic to the spinal cord. Le Gros Clark (1958) has suggested 'that the lower sensory centres of the central nervous system function as sorting stations' and 'that the regrouping of somatic sensory fibres (and of the impulses conveyed by them) as they enter the nervous system must be determined by some sort of positive selectivity exerted by the different groups of receiving cells in the lower sensory centres—that is to say, the analysis of the sensory inflow must depend primarily on their activity'. The fact that tubocurarine applied to the upper cervical cord produces a pattern of spontaneous movements which is that of the scratch reflex is compatible with this concept, in that the lower sensory centres, when exposed to drug stimulation, exhibit this property of 'selective sorting'.

SUMMARY

1. Application of tubocurarine to the dorsal surface of the cervical cord of cats produced both spontaneous scratching movements and facilitation of the scratch reflex elicited by rubbing the pinna and the skin behind the ears. The area of application from which these effects were obtained

extended from the level of C1 down the cord to the level of C2. At this lower level a stronger concentration of tubocurarine was required.

2. The pattern of movements produced by tubocurarine in decerebrate animals was usually more sustained than that seen in cats anaesthetized with pentobarbitone sodium. In both conditions these movements were decreased in strength by the addition of pentobarbitone sodium. The addition of chloralose abolished them.

3. Transection of the medulla below the obex greatly diminished the strength of the movements.

REFERENCES

FELDBERG, W. & FLEISCHHAUER, K. (1960). Scratching movements evoked by drugs applied to the upper cervical cord. *J. Physiol.* **151**, 502—517.

FELDBERG, W. & SHERWOOD, S. L. (1954). Injections of drugs into the lateral ventricle of the cat. *J. Physiol.* **123**, 148–167.

GERARD, M. W. (1923). Afferent impulses of the trigeminal nerve. The intramedullary course of the painful, thermal and tactile impulses. *Arch. Neurol. Psychiat.* **9**, 306–338.

LE GROS CLARK, W. (1958). Sensory experience and brain structure. *J. ment. Sci.* **104**, 1–13.

MERLIS, J. K. (1940). The effect of changes in the calcium content of the cerebrospinal fluid on spinal reflex activity in the dog. *Amer. J. Physiol.* **131**, 67–72.

SHERRINGTON, C. S. (1908–9). A mammalian spinal preparation. *J. Physiol.* **38**, 375–383.

SHERRINGTON, C. S. (1910). Notes on the scratch reflex of the cat. *Quart. J. exp. Physiol.* **3**, 213–220.

SHERRINGTON, C. S. (1917). Reflexes elicitable in the cat from pinna, vibrissae and jaws. *J. Physiol.* **51**, 404–431.

J. Physiol. (1960), **153**, *pp.* 52–73
With 13 *text-figures*
Printed in Great Britain

NEURONE ACTIVITY IN THE CAT'S TRIGEMINAL MAIN SENSORY NUCLEUS ELICITED BY GRADED AFFERENT STIMULATION

By I. DARIAN-SMITH*

From the Department of Physiology, University of Sydney, Sydney, Australia

(*Received* 26 *February* 1960)

Previous studies of the synaptic mechanism in afferent projection pathways have demonstrated two features which differ from those observed in the activation of the motoneurone (Sherrington, 1929; Coombs, Eccles & Fatt, 1955; Hunt, 1955). These are the powerful synaptic excitatory effect of a single presynaptic impulse, and the very limited convergence occurring at the synaptic relay. Amassian & de Vito (1957) have shown that a single impulse is sufficient to discharge cells in the cuneate nucleus repetitively, a region in which Glees & Soler (1951) had previously demonstrated convergence of only a few fibres on to each nerve cell. Similar features characterize the synaptic excitation of cells in Clarke's column by Group Ia fibres (Laporte, Lundberg & Oscarsson, 1956), and also of cells in the lateral geniculate body (Bishop, Jeremy & McLeod, 1953) activated by optic nerve fibres.

The modification of a simple presynaptic volley occurring at the synaptic relay within the main sensory nucleus of the trigeminal nerve has been studied in the present experiments. The responses of single nerve cells to graded afferent volleys have been examined in order to make a quantitative estimate of the distribution of the threshold for excitation of the cell; the associated latency changes have also been studied. The nature of the convergence of presynaptic fibres on the trigeminal neurones was further investigated by examining the effect of varying the cutaneous stimulus site within the receptive field of a cell on its evoked discharge.

METHODS

Adult cats weighing 2·5–3·5 kg were used for all experiments. They were anaesthetized with an initial intravenous injection (0·5 ml./kg body wt.) of sodium pentobarbital ('Sagatal'; May and Baker, 64 mg/ml.) sufficient to carry out operative procedures. Anaesthesia was maintained during the remainder of the experiment by supplements of this anaesthetic 0·1–0·2 ml./hr. This dosage was sufficient to maintain light surgical anaesthesia. To reduce

* Senior Research Fellow, National Health and Medical Research Council, Australia.

respiratory pulsation of the brain stem during the recording period modified diffusion respiration was used. The relaxant gallamine triethiodide (Flaxedil; May and Baker) was administered intravenously (2 mg/kg body wt.) every 20–25 min. Intermittent injection of the short-acting Flaxedil permitted adequate control of the barbiturate anaesthesia. The respiratory excursion was reduced to 20–25 ml./stroke. Arterial oxygenation was attained by the continuous administration of intratracheal oxygen, and carbon dioxide elimination by increasing the respiratory rate (26/min). The arterial oxygen saturation and carbon dioxide content were thus maintained at normal levels, being checked in several of the early experiments by blood gas analysis.

The head of the animal was fixed in a stereotaxic apparatus, unilateral occipital craniotomy performed, and the dura overlying the cerebellum removed. Owing to the position of the main sensory nucleus of the trigeminal nerve, high under the bony tentorium cerebelli, recording electrode penetrations were made at an angle of 30° to the vertical. A circular Perspex chamber was attached to the skull over the craniotomy opening with dental impression compound; a sliding window on its upper surface moved at right angles in all directions to the penetrating electrode (Erulkar, Rose & Davies, 1956). The electrode penetrated the window through a small liquid-tight gland.

Tungsten micro-electrodes (Hubel, 1957) with a tip diameter of 1–2 μ and resistance to direct current of 2–5 MΩ were used for unit recording. The reference lead was a platinum plate in close contact with the skin flap of the scalp.

The criteria used for assessing the site of recording were (*a*) the position of the electrode track seen in serial Nissl-stained sections (Einarson's gallocyanin method, 1932), and (*b*) the form of the evoked field response. Histological identification of the electrode track fixed the lateral and rostrocaudal position of the penetration. The evoked field potential was useful in defining the medial and inferior limits of the nucleus, because a sharp reversal of the post-synaptic negative potential occurred at these borders; however, considerable field spread occurred dorsally.

A capacity-coupled amplifier was used. Records were photographed with a Grass kymograph camera attached to a double-beam oscilloscope. In all records a downward deflexion indicated a positive change in potential at the electrode tip.

The receptive field of each cell was mapped over the ipsilateral clipped upper lip with a fine camel-hair brush. Latency changes and the firing pattern of the cell were then examined by electrical stimulation of the skin. A battery of 6 bipolar steel electrodes (inter-polar distance 2 mm) was positioned 2–3 mm above the edge of the upper lip parallel to it. Succeeding pairs of electrodes were 2 mm apart. The stimulus current could be separately directed to each pair of electrodes. The stimulus duration was 100 μsec for all experiments.

No significant fall in blood pressure, which was checked hourly, occurred during the recording period. Mean blood pressure for the whole series of experiments was 130 mm Hg (S.D. = $\pm$15 mm Hg).

Estimation of the distribution of the threshold for excitation of single neurones

If the discharge of a neurone evoked by a near-threshold stimulus is examined repeatedly, it is observed that the unit fires in response to a proportion only of the stimuli. This results from the fact that there is a continuous fluctuation in the threshold of the cell, inherent to the phenomenon of excitation. The cell fires whenever its threshold falls to the input level and the proportion of stimuli which evoke discharge of the nerve cell is an estimate of the probability that the threshold of the unit will fall to or below this level. By determining the probability of firing for input levels over the whole of the threshold range, the distribution function of the threshold for excitation may be estimated.

For each nerve cell isolated the probability of firing was determined at 4–12 stimulus levels and from these the distribution function of the threshold was estimated. Fifty responses were examined at each input level (interval between succeeding stimuli 1 sec).

The sigmoid curve relating the probability of firing and the stimulus intensity approximated a normal distribution function for all the neurones investigated. For units discharging repetitively distribution functions were constructed for each spike in the discharge; these also were approximately normal.

For convenience in analysis of these distribution functions of the threshold for excitation, use was made of the probit transformation of the probability of firing at each input level (Finney, 1952).

The effect of this transformation was that the relation between the probability of firing and the applied stimulus could then be expressed linearly and consequently defined by two parameters: (*a*) the median threshold, which was that stimulus intensity at which the probability of firing of the particular action potential was 0·50, and (*b*) the variance of the threshold distribution which defined its dispersion

$$\left(\text{variance} = \frac{1}{(\text{slope of probit line})^2}\right).$$

These parameters were estimated either graphically or arithmetically.

RESULTS

The input to the main sensory nucleus of the trigeminal nerve

In the evoked potential recorded with a tungsten micro-electrode in the main sensory nucleus of the trigeminal nerve, field and unit responses were obtained without marked attenuation of the former. This did not impair unit recording, as most neurones were maximally activated with a low input to the nucleus; as a result, the unit responses usually had a much higher amplitude than the field potential. The evoked field response recorded in the main sensory nucleus on electrically stimulating the skin was characteristic of this region of the nuclear complex; it consisted of a synchronous, diphasic, initially positive component, and a negative component lasting 2–4 msec. With strong stimulation this was followed by a low-amplitude positive potential lasting 5–10 msec. With repetitive stimulation at a frequency of 750 c/s the initial diphasic component was unaffected, indicating its presynaptic nature. The conduction velocity of the activated fibres, estimated from the latency of the commencement of the negative component of the presynaptic spike (not allowing for delay at the stimulus site), was 71 and 75 m/sec respectively, in two experiments. The length of the peripheral path varied between 5·5 and 7·0 cm.

When comparing the estimated threshold distributions of a neurone for different sites of peripheral stimulation, a possible source of error is the varying effectiveness of the contact of the stimulating electrodes with the skin. Equal voltages applied to the skin might not be equally effective in stimulating cutaneous nerve fibres. To check this, the relationship between the stimulus voltage applied to the skin and the amplitude of the presynaptic spike was determined for a series of closely apposed stimulus sites. This relationship is shown in Fig. 1 for three separate regions. The error was small, as is shown by the limited scatter of the results. The relation-

ship was approximately linear, with only slight flattening of the curve at the highest voltages, indicating that the maximal electrical stimulus used in the present experiments did not activate all the fibres projecting from the stimulus site to the main sensory nucleus.

Some measure was also obtained of the extent of the lateral spread of effective stimulating current about the electrodes applied to the skin. This lateral spread was certainly less than 2 mm and probably less than 1 mm for the maximal stimulus intensity used (106 V). Six neurones had a receptive field whose diameter was less than 4 mm. They were activated

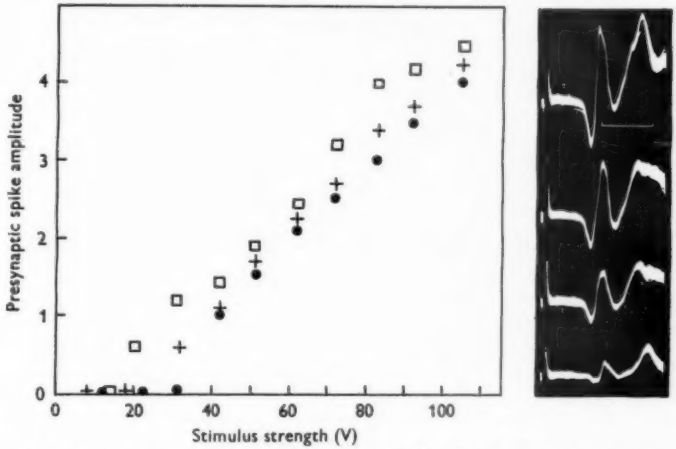


Fig. 1. Relation between the voltage applied to the skin and the peak-to-peak amplitude of the evoked presynaptic spike. The relation has been determined for three peripheral stimulus sites (□, +, ●) on the upper lip. The distance between successive stimulus sites was 2 mm. It is seen that the stimulus site did not greatly influence the relationship. The records (40 superimposed traces) illustrate the simple character of the presynaptic spike at various stimulus intensities. Time marker, 1 msec.

by one pair of bipolar electrodes within the field, and at that point the median threshold was quite low (mean value 34·4 V). However, no excitation occurred on stimulating the skin 2 mm away with the neighbouring electrodes, even with the highest voltage available.

General characteristics of single neurone activity within the main sensory nucleus

In these experiments the evoked activity of 120 neurones was investigated. Many more neurones were observed in the nucleus which fired 'spontaneously', but whose discharge pattern was not obviously related

to the cutaneous stimulus. In the units investigated 'spontaneous' firing with frequencies up to 10/sec was usually present.

The action potentials of the units recorded extracellularly displayed the variation in polarity observed previously in other cellular regions within the central nervous system (Kuffler, 1953; Rose & Mountcastle, 1954; Svaetichin, 1958). The action potentials of forty-five neurones were initially negative, monophasic or diphasic spikes, sixty-one were initially positive and diphasic, and fourteen units monophasic and positive. These action potentials were probably recorded extracellularly in the region of the cell

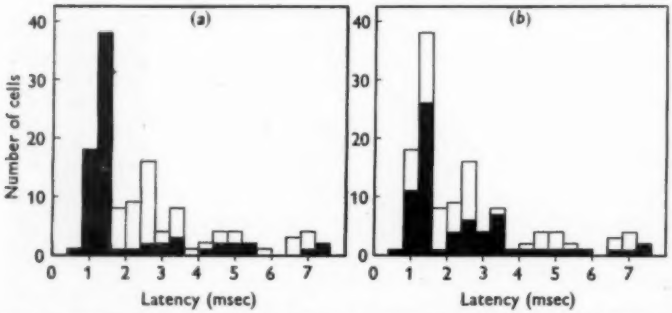


Fig. 2. Frequency distribution of the shortest mean latency of trigeminal neurones. In *a* the frequency distribution has been subdivided according to the polarity of cells; ■, distribution of neurones with initially positive action potential; □, neurones with initially negative action potentials. In *b* the same frequency distribution has been subdivided on the basis of the discharge pattern: ■, non-repetitively firing units; □, repetitively firing units.

body, except for those units when a positive monophasic spike only was recorded; the electrode tip may well then have been in the region of the axon of the neurone (Tasaki, Polley & Orrego, 1954; Freygang, 1958). Reversal of the polarity of the action potential from initial negativity to initial positivity was seen only occasionally following slight movement of the electrode tip. The initially negative action potentials whose threshold characteristics were very stable over a long period of recording had amplitudes of 1–2 mV. The initially positive spikes had larger amplitudes (1–6 mV) but responses were more variable over long periods of observation.

Figure 2*a* is a histogram showing the frequency distribution of units grouped according to the mean latency of the evoked potential at the highest stimulus strength used and applied to the central part of the neurone's repetitive field (i.e. the shortest latency). Fifty-four of the units examined had a latency of 1·60 msec or less. The action potential of all these short-latency units was initially positive. The longer-latency units, with a small peak at 2·4–2·8 msec, were mainly initially negative.

This distribution of latencies may also be considered from the viewpoint of the nature of the evoked discharge. Most non-repetitively firing units were of short latency (1·0–1·6 msec), with a second peak about 2·8 msec. The repetitively firing units more commonly had a longer latency (Fig. 2*b*). This subdivision was less sharp, however, than that seen with the cell polarity distribution.

The delay between the arrival of the presynaptic volley at the nucleus (latency of the commencement of the negative component of the presynaptic spike 0·90 msec; S.D. = ±0·15 msec) and the firing of units of

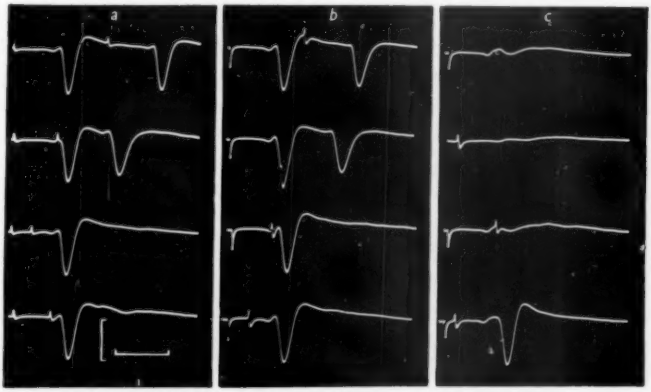


Fig. 3. *a*, Interaction of conditioning and test stimuli of equal supramaximal intensity applied to the same point within the receptive field of a monosynaptically activated trigeminal neurone; block of the test response occurred at a stimulus interval of 1·05 msec. *b*, Interaction of conditioning and test stimuli (both supramaximal) applied to two regions within the receptive field of the neurone, 8 mm apart; block of the test response occurred at a stimulus interval of 1·12 msec. *c*, Interaction of conditioning and test stimulus applied to two regions within the receptive field, 8 mm apart. Each separate stimulus evoked no propagated response; spatial summation of the synaptic activity of the arriving volleys occurred, however, when the stimulus interval was less than 0·35 msec; this is illustrated in the lowest tracing. Voltage (5 mV) and time calibrations (1 msec) are indicated by the vertical and horizontal bars respectively.

the short-latency group was sufficient for the latter to be monosynaptically activated. Figure 3 illustrates the post-synaptic nature of discharge of such a neurone. Figure 3*a*, *b* illustrates the blocking interaction of the discharge evoked by two supramaximal stimuli applied to the same, and widely separated, regions respectively within the receptive field of the unit. The refractory period was so short that no distinction between pre- and post-synaptic activation could be made. However, in Fig. 3*c* summation of two subthreshold stimuli applied to widely separated regions

of the receptive field did result in discharge of the cell. This phenomenon of spatial summation is post-synaptic.

It will be seen that the sample of trigeminal neurones consisted of two main groups: one group, monosynaptically activated, not firing repetitively, and with action potentials all of which were initially positive; and a later polysynaptically activated group of cells, with varying discharge patterns, which included most of the repetitively firing neurones.

The absolute refractory period following discharge of the monosynaptically activated neurones was determined for eight units. The mean value was 0·88 msec (S.D. = ±0·13 msec). Of the eighty-four units tested seventy-six responded to light mechanical stimulation of the skin in the region from which they were evoked electrically, indicating the mechanoreceptor cutaneous terminals of these fibres. The absolute refractory period following mechanical stimulation of such a cutaneous terminal appears to be of the order of 2 msec or more (Gray & Malcolm, 1950; Lindblom, 1958) suggesting that the site of activation of the cutaneous fibres by a shock stimulus was proximal to the receptor ending.

Single neurone responses evoked by graded afferent stimulation of the central region of the receptive field

The discharge pattern of trigeminal neurones evoked by cutaneous stimulation was modified by two factors, namely, the stimulus intensity and the stimulus site within the receptive field of the particular unit. In order to compare the response evoked by increasing stimulus intensity in different neurones, the stimulus site was first fixed as the region of lowest median threshold within the receptive field. With some of these units the additional effect of varying the stimulus site within the receptive field was then examined.

Monosynaptically activated neurones. The distribution functions of the threshold for excitation of the monosynaptically activated non-repetitively firing units were uniform. Most units had a median threshold value (when stimulated from the central part of the receptive field) below 30 V. The dispersion of the threshold distributions of these cells, estimated by the variance of this distribution for each cell, was small, the mean value being 0·63 V (i.e. the S.D. of the threshold distribution, $\sqrt{0{\cdot}63} = \pm 0{\cdot}79$ V). Thus 95% of threshold values fell within $0{\cdot}79 \times 2 = 1{\cdot}58$ V of the median threshold. Over the threshold range of stimulation the latency of monosynaptically activated units varied slightly, increasing by 0·15–0·20 msec at the lowest input levels.

A group of eight cells, monosynaptically activated, fired repetitively to a synchronous afferent volley. The pattern of discharge was identical for all cells in the group, the evoked response always consisting of two

action potentials, which could not be differentially fired by varying the stimulus intensity. The median thresholds for the individual neurones were scattered throughout the whole input range, but the variance of the threshold distribution (identical for both spikes) for each unit was uniformly small, the mean value being 0·61 V (S.D. = ±0·78 V). The mean latency of the first spike was 1·25 msec (S.D. = ±0·15 msec), being followed by a negative component lasting several milliseconds. The second spike was superimposed on the negative component, the mean interspike interval being 1·25 msec (S.D. = ±0·26 msec). The development of this type of discharge pattern in non-repetitively firing units was observed on several occasions coincident with movement of the electrode tip nearer the cell. This suggests that such a discharge pattern may result from local injury of the neurone due to the electrode.

Polysynaptically activated neurones, non-repetitively firing

The median thresholds of non-repetitively discharging units with longer latencies were of the same order as those of monosynaptically activated units. The variance of the threshold distribution, however, was greatly increased for most of these cells, often being 300–400 times greater than the variance of monosynaptically activated neurones. This is shown in Fig. 4, where the variance of the threshold distribution has been plotted against the latency for all non-repetitively firing units.

Much greater variability in the latency was also observed than in monosynaptically activated units, a graded reduction occurring on increasing stimulus intensity. Quantal changes in latency were not observed when the stimulus site was near the centre of the receptive field.

Polysynaptically activated neurones, repetitively firing

Usually the discharge pattern of a neurone with the maximal number of spikes was evoked by the highest stimulus strength used. In Table 1 the frequency distribution of the maximal spike number in the evoked discharge is given. The two-spike discharge was by far the commonest repetitive discharge observed.

The threshold distribution. A graded change was observed in the threshold distributions of the successive spikes of the repetitive discharge of the trigeminal neurones investigated. In Fig. 5*a* the relation between the probability of firing and the stimulus intensity is shown for successive spikes in a seven-spike repetitive discharge. In Fig. 5*b* the median threshold (upper graph) and the variance of the threshold distribution (lower graph) of successive spikes is shown. Similar increases in these parameters of the threshold distribution were observed for other neurones with more limited spike sequences. The threshold distribution functions

of a unit with a maximal discharge pattern of two spikes are shown in Fig. 6.

The total stimulus range over which a graded change in the discharge pattern was observed for any one unit is defined by the threshold distribution functions of the first and the last spike in the sequence. For the cell

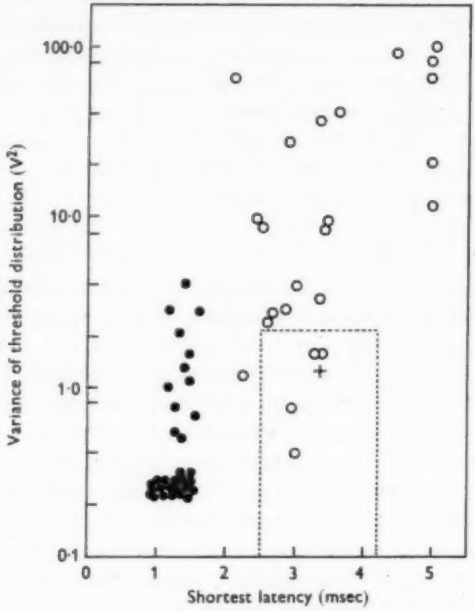


Fig. 4. Relation between shortest latency of non-repetitively firing neurones and variance of threshold distribution. ●, neurones activated monosynaptically; ○, neurones activated polysynaptically; +, mean value for variance and latencies of the first spike in the repetitive discharge of 25 cells. The interrupted lines indicate the standard deviations of the mean latency and variance respectively. Semi-log scale.

TABLE 1. Distribution of maximal number of spikes in repetitive discharge

	No. of spikes in discharge								
	1	2	3	4	5	6	7	8	9
No. of neurones	60	37	9	9	3		1	1	1

illustrated in Fig. 5 a graded response occurred over the whole range of input levels tested. On the other hand, for a two-spike sequence such as is shown in Fig. 6 a graded response occurred only over the range defined by the two curves in the graph. Even for a two-spike train the range of inputs over which a graded response was observed varied in different

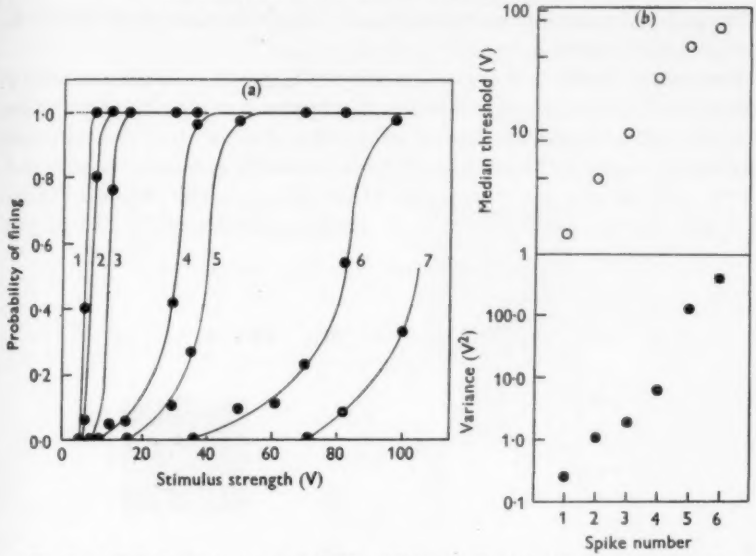


Fig. 5. *a*, Relation between probability of firing (ordinate) and stimulus strength (abscissa) for successive spikes in a repetitive discharge; the threshold distributions of successive spikes are numbered 1, ..., 7. *b*, Parameters of the threshold distributions of successive spikes in the discharge: the abscissa indicates the position of the spike in the sequence; in the upper graph the ordinate is the median threshold (V) and in the lower the ordinate is the variance of the threshold distribution (V^2), log. scale.

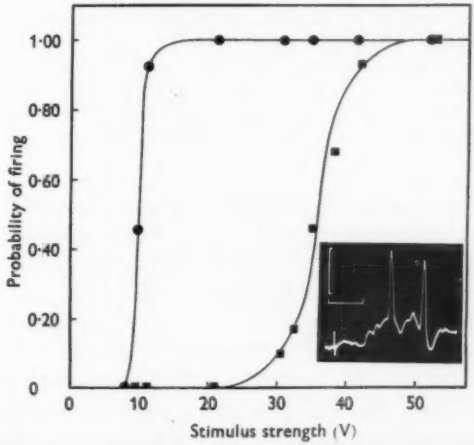


Fig. 6. Relation between the probability of firing (ordinate) and the applied stimulus (abscissa) for a neurone with a maximum discharge pattern of two spikes. The record of a typical evoked response is shown in the insert, with voltage (1 mV) and time (1 msec) calibrations represented by vertical and horizontal bars respectively. ●, probability of firing of 1st spike; ■, probability of firing of 2nd spike.

units, and in the same unit when activated from different regions within the receptive field of the neurone.

From most units, once the probability of firing of a particular spike in a repetitive discharge had reached unity, further increases in the stimulus strength did not affect its firing rate. However, with a few neurones further increases in the applied stimulus caused a reduction in the probability of firing of some components of the disharge. The response pattern of such a neurone is shown in Fig. 7. Between input levels of 25 and 55 V

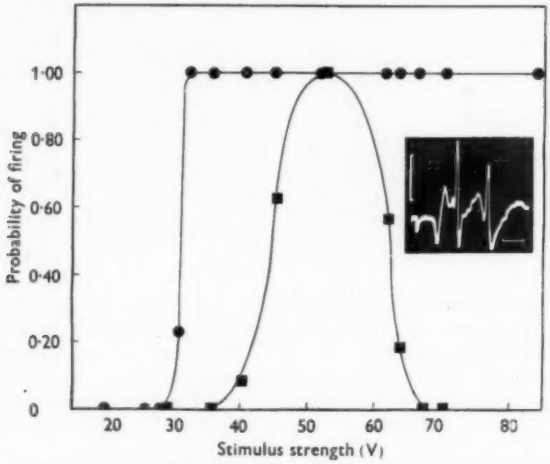


Fig. 7. Graph of the stimulus–response (probability of firing) curves for a neurone where a reduction in the probability of firing occurred with high input levels. The firing rate of the second spike (■) after reaching a maximum fell back to zero at high input levels, whilst the earlier spike (●) continued firing with a probability of unity. The inset is a record of typical evoked potentials; voltage (1 mV) and time (1 msec) calibrations indicated by the vertical and horizontal bars respectively.

the relation between stimulus strength and probability of firing was sigmoid for the two spikes of the train. Above this input level the shorter-latency spike continued to fire with a probability of 1·0, whereas the probability of firing of the second spike fell back to zero at input levels above 75 V. In a few units with a longer repetitive discharge the probability of firing of the last two or three spikes in the discharge fell off at the highest stimulus intensities. No unit was observed in which the probability of firing of the first spike in a repetitive discharge was reduced on increasing the stimulus strength; nor was the probability of firing of any non-repetitively firing unit reduced at high input levels to the nucleus.

Latency of discharge. Simultaneously with the rise in the probability of

firing of the successive spikes in the response of a repetitively firing neurone, considerable changes occurred in the latencies of these spikes. For a two-spike train by far the most common pattern of distribution of latencies of these spikes is illustrated in Fig. 8. At input levels just sufficient to activate the unit the first spike appeared with quite a long latency, with a peak in the distribution about 4·6 msec. With only

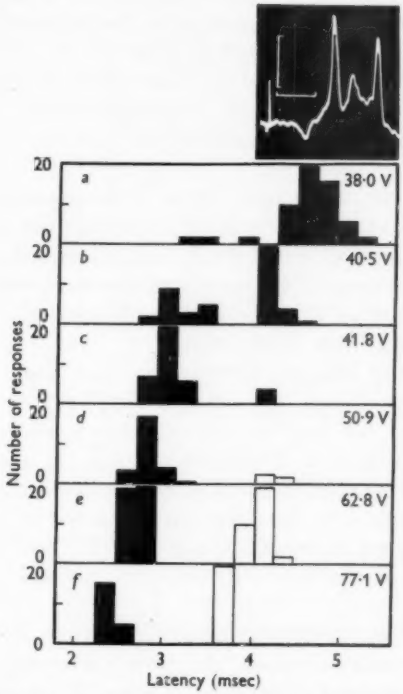


Fig. 8. Changes in the latencies of the two spikes in a repetitive discharge on increasing the stimulus intensity. The frequency distribution of the latency of discharge of each spike at a constant stimulus level is shown in each histogram. The stimulus strength used for each frequency distribution is indicated on the right side in volts, being lowest for the top graph. ■, frequency distribution of the low-threshold spike; □, that of the higher-threshold spike.

slight increases in the input the latency of this spike abruptly shortened as seen in Fig. 8*b*. This change was not graded but involved a sudden reduction in latency of about 1·3 msec. The bimodal distribution of the latency of this first spike is seen in Fig. 8*b*. With a slight increase in input nearly all the spikes fired with a short latency of 2·8 msec (Fig. 8*c*). Further increases in the applied stimulus caused now only a very small

reduction in the latency of the first spike, and with those responses in which the latency of the first spike was 2·4 msec a second spike now developed (Fig. 8*d*). The latency of this spike approximated that observed for the first spike at low input levels. With a further increase in the input level, the latency of most of the first spikes fell into the 2·4 msec group and simultaneously the probability of firing of the second spike rose. A further small reduction in the latency of the first spike at the highest input level is shown in Fig. 8*e*. Some shortening of the interspike interval was observed. Latency changes of this type were observed in thirty-three of the thirty-seven units which had a maximal discharge pattern combining two spikes. In the four cells which did not fire in this manner, no discontinuity was observed in the latency changes on increasing the stimulus intensity.

Neurones firing with a discharge pattern containing more than two spikes displayed similar changes in latency on increasing the afferent stimulus. As the input was increased, quantal reductions amounting to 1·5–2·0 msec in the latency of discharge of the early spikes were observed, and in addition there was a gradual shortening of the latency of each spike. This however, was never greater than 1 msec.

Comparison of the relation between the variance of the threshold distribution and the latency of the first spike of repetitively firing units with that observed for the long-latency, non-repetitively firing units demonstrated one difference, illustrated in Fig. 4. It is seen in this graph that the dispersion of the threshold distribution for the first spike of a train frequently was very low, and of the order observed for monosynaptically activated neurones, which did not fire repetitively. This occurred in spite of the long latency, and presumably polysynaptic preceding pathway.

Time interval between successive spikes in a repetitive discharge. Observations made concerning the effect of varying stimulus strength on the interspike interval agreed with those previously reported by Rose & Mountcastle (1954). With a decrease in input level over the threshold range there was a slight but significant increase in this interval. Thus, for the group of thirty-seven cells with a maximum repetitive discharge of two spikes the mean interval was 1·62 msec (S.D. = $\pm$0·41 msec) when the probability of firing was unity. When the input for these cells was reduced and the probability of firing was 0·20–0·30, the interspike interval increased significantly by 0·22 msec (S.D. = $\pm$0·05 msec). Figure 8 illustrates such an increase. Similar observations were made with longer repetitive discharges. In Table 2 latency changes are tabulated over a wide input range for a cell firing maximally with a discharge of seven spikes. It will also be noticed that for this neurone at any one input level, in discharges

of a constant number of spikes, there was a progressive increase in the time interval between succeeding spikes. This was a common finding, but not the invariable pattern. Sometimes either the shortest or longest mean interspike interval occurred in the central part of the discharge (cf. Rose & Mountcastle, 1954).

In the records of the repetitive discharges of some trigeminal neurones an inflexion at the commencement of each action potential, suggestive of separate synaptic potentials, was seen. Typical evoked discharges are shown in Fig. 9.

TABLE 2. Time interval between succeeding spikes in the repetitive discharge of a trigeminal neurone at different input levels to the nucleus

Stimulus strength (V)	No. of observations	Latency of 1st spike (msec)	Spike intervals (msec)						Mean duration of train (msec)
			I_1	I_2	I_3	I_4	I_5	I_6	
10·8	9	6·70	1·94	—	—	—	—	—	1·94
	30	6·37	1·92	2·52	—	—	—	—	4·44
24·1	20	5·92	1·83	2·65	—	—	—	—	4·48
	18	5·76	1·69	2·10	4·45	—	—	—	8·24
35·1	30	5·62	1·84	2·64	4·15	—	—	—	8·64
	9	4·54	1·63	1·97	3·08	3·68	—	—	10·36
71·1	34	5·20	1·63	1·92	2·41	3·03	—	—	8·99
	4	4·96	1·62	1·92	2·09	2·62	3·41	—	11·66
105·8	24	4·90	1·62	1·70	1·98	2·18	2·37	—	9·85
	12	4·85	1·60	1·73	1·90	2·02	2·18	2·48	11·91

First column indicates the stimulus intensity (volts) applied to the skin. Second column indicates the number of observations made, of which the tabulated figures are the mean. At each input level there are two entries because the total number of spikes in the discharge varied with succeeding stimuli. I_1, I_2, ... I_6 are the mean time intervals between the earliest and second spike, second and third spike ... sixth and seventh spike, in the discharge.

Single-neurone responses evoked by graded afferent stimulation of different regions of the receptive field

Excluding six neurones, whose receptive fields extended over one half or more of the ipsilateral face, the size of the cutaneous receptive fields (ipsilateral upper lip) of neurones in the main sensory nucleus was fairly uniform. The mean diameter parallel to the lip margin of the receptive fields of sixty-eight such neurones was 12·4 mm (S.D. = ±6·2 mm).

Threshold distribution. Comparison of the threshold distributions of a single neurone determined for several stimulus sites within the cell's receptive field demonstrated a progressive change in their characteristics. Both the median threshold and the dispersion of the threshold distribution increased in a graded manner as the stimulus site was moved towards the edge of the receptive field. No discontinuity was observed in the receptive fields of any of the neurones investigated. Figure 10*a* shows the progressive rise in the median threshold at the edge of the receptive field of several

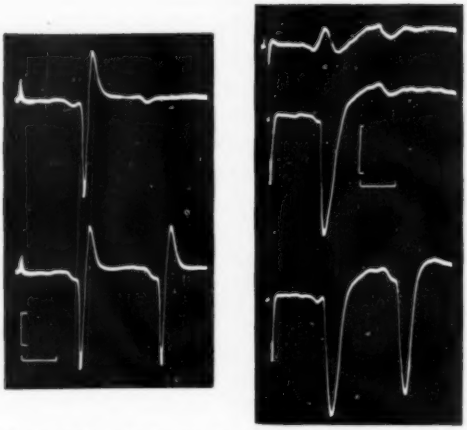


Fig. 9. Evoked repetitive discharges from two neurones demonstrating separate synaptic potentials for each spike in the discharge. For both cells when the second spike failed to discharge a small synaptic potential was still evoked. Voltage (1 mV) and time (1 msec) calibrations are indicated by the vertical and horizontal bars respectively.

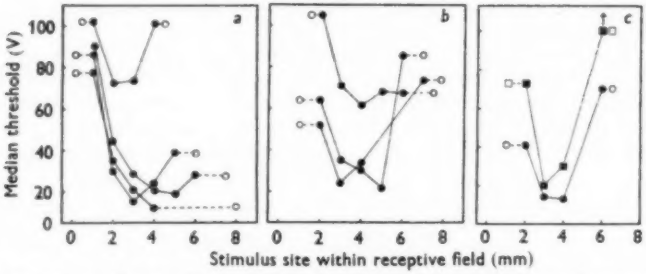


Fig. 10. The median threshold at different positions within the neurone's receptive field. Ordinate, median threshold (V); abscissa, intervals across one diameter of receptive field (mm). In *a* are shown changes within the receptive fields of monosynaptically activated cells. In *b* the cells were polysynaptically activated. In *c* changes in the median threshold of the first (●) and second (■) spikes of a repetitively firing unit are shown; ○, □ indicate the edge of the receptive field.

monosynaptically activated units. The threshold changes observed in polysynaptically activated units were similar, as is shown in Fig. 10*b*.

Figure 11 illustrates the changes observed in the dispersion of the threshold distribution at different sites of stimulation within the receptive field. With monosynaptically activated neurones the increase in the dispersion, estimated by the variance of the distribution, occurred only in the peripheral 2–3 mm. With units of longer latency the increase in the

dispersion at the edge of the receptive field was quantitatively much larger, as is seen in Fig. 11*b*. Graded changes in the threshold distribution were also observed with repetitively firing neurones. For any particular spike in the repetitive discharge both the median threshold and the variability of the threshold increased progressively towards the edge of the field. The changes in the median thresholds are shown in Fig. 11*c* for a two-spike discharge.

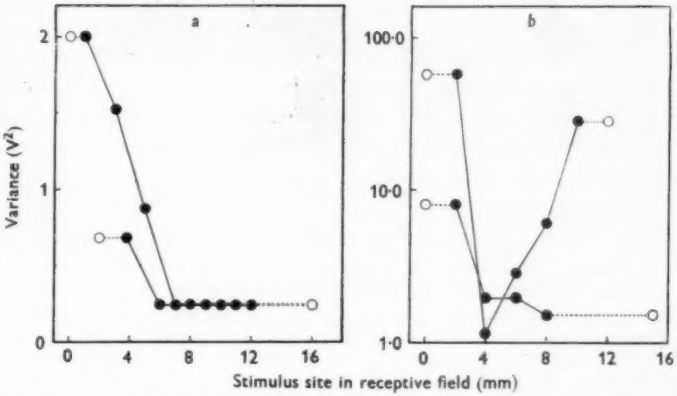


Fig. 11. *a*, The variance of the threshold distribution at different positions across the receptive fields of two monosynaptically activated neurones; on the right the threshold distribution was not determined, but the limit of the field was defined. Ordinate, variance of threshold distribution (V^2). Abscissa, stimulus site along one diameter of receptive field (mm). *b*, Variance of threshold distribution within the receptive fields of two non-repetitively firing polysynaptically activated units; the ordinate scale is logarithmic.

Latency of discharge. Monosynaptically activated neurones displayed no more than 0·15–2·20 msec variation in the latency of firing from any one site of stimulation within the receptive field, nor was there any significant difference in the latency of discharge when stimulated from central or peripheral regions in the receptive field. This was true also for longer-latency non-repetitively firing units. However, for a few such cells there were considerable differences in the latency of discharge from different regions of the receptive field. As is seen in Fig. 12*a*, the commonly observed graded reduction in latency of discharge occurred when the site of stimulation within the receptive field was central. When the neurone was stimulated from the edge of the receptive field, in addition to this small graded effect a large discontinuous reduction also occurred with an increase in stimulus strength, the quantal reduction being about 1·5 msec (Fig. 12*b*).

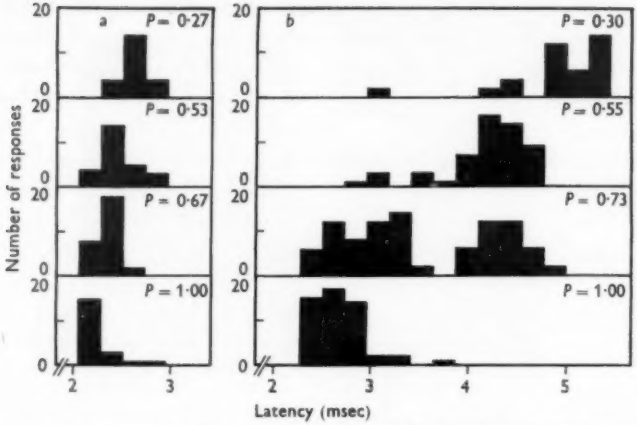


Fig. 12. *a*, Histograms showing the frequency distribution of the latency of discharge of a non-repetitively firing trigeminal neurone at increasing stimulus intensities; the stimulus site was in the central part of the receptive field. The probability of firing (*P*) as an estimate of input level is indicated for each histogram; a small graded reduction of about 0·5 msec is shown as the input was increased. *b*, The stimulus site was 1 mm from the edge of the receptive field: for corresponding histograms the probability of firing was approximately the same as in *a*. In addition to a small gradual reduction such as was observed in *a* there was a large quantal reduction of approximately 1·5 msec.

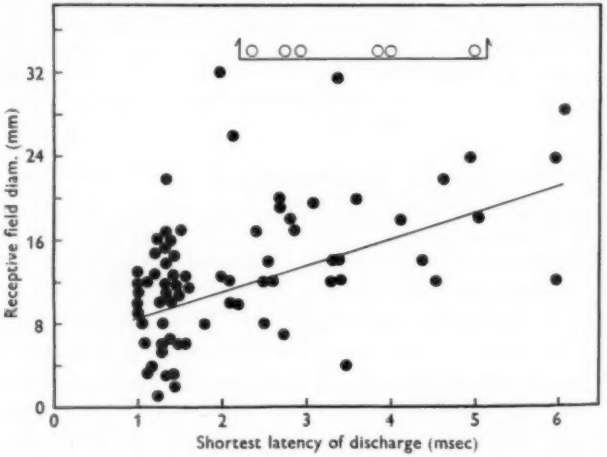


Fig. 13. Relation between the shortest mean latency of a trigeminal neurone and the diameter of its receptive field. The linear regression line (calculated for cells represented by ●) indicates the average rate of increase in the diameter of the receptive field with increasing latency. ○, separate group of units with very large receptive fields (not included in the calculation of regression line).

Increases in the peripheral stimulus intensity caused an abrupt reduction in the latency of repetitively discharging neurones, regardless of the stimulus site within the receptive field. The only differences were that stimulus intensities at which these latency changes occurred were higher in the periphery of the receptive field than in the central part.

The diameter of the receptive field of a neurone was related to its latency, as is shown in Fig. 13. In spite of a fairly wide scatter of values there was a significant increase in the receptive field diameter for units with longer latencies. The linear regression line, significant at the 1 % level, shows the average increase for the main group of cells. Those six cells with very large receptive fields were not included in the estimation of the regression line, as these would have influenced it unduly. For each increase in latency of 1 msec there was an average increase in the diameter of the receptive field by 2·4 mm. This relationship suggests that there was increasing convergence occurring within the nucleus as the preceding pathway to the unit included additional synapses.

DISCUSSION

In the present experiments the activity of two groups of neurones was recorded in the trigeminal main sensory nucleus. One group was monosynaptically activated and discharged with a single action potential unless the cell was injured; the other group of cells was presumed to be polysynaptically activated and the discharge pattern was varied but commonly repetitive. It is generally agreed that most long ascending fibres from the main sensory nucleus project to the arcuate nucleus of the thalamus (Winkler, 1921; Torvik, 1957). The latency of the earliest potential evoked in the contralateral arcuate nucleus, following electrical stimulation of the upper lip, is approximately 2·0 msec (unpublished results); hence the axons of at least some of the monosynaptically activated neurones in the main sensory nucleus must have projected to the thalamus.

The pathways for the presumed polysynaptic discharge of neurones within the main sensory nucleus remain uncertain. Åström (1952) observed cells in Golgi preparations of the nucleus of the mouse which could possibly be interneurones, but Torvik (1957) concluded from studies of retrograde degeneration in the cat that all cells within the nucleus have long axons projecting at least to the level of the mid-brain. Possible pathways for late activation of second-order neurones within the nucleus may be recurrent collaterals arising from the long ascending axons; these have been described by Cajal (1909) in the cuneate nucleus and other sensory relays. Convergence on polysynaptically activated neurones was greater than that on monosynaptically activated cells: this is evident from the observation that the cutaneous receptive fields of trigeminal

cells were larger for the polysynaptically activated cells. However, the receptive fields were only slightly increased, and might well have resulted from converging recurrent collaterals of neighbouring cells. Amassian & de Vito (1957) considered recurrent collaterals to be the anatomical pathway involved in the after-discharge observed in the cuneate nucleus.

The estimations of the distributions of the threshold for the excitation of trigeminal neurones demonstrated that even when the stimulus site within the respective receptive fields was centrally placed, considerable variation existed between cells. The threshold distributions of monosynaptically activated sensory neurones had dispersions more comparable with those of peripheral nerve fibres (Pecher, 1939) than with those of the monosynaptically activated motoneurones (Hunt, 1955). This very small dispersion of the threshold distribution of such trigeminal neurones implies that the activation of only a few peripheral fibres converging on the cell was necessary for its discharge. Activation of at least two fibres was, however, necessary for discharge of the cell, as was shown by the summation of the synaptic action of two subthreshold volleys converging on the cell from widely separated areas within the receptive field.

With the longer latency non-repetitively firing neurones, the considerable increase in the dispersion of the threshold distribution suggests that either the activation of many converging fibres was necessary to generate an action potential, or, more probably, that considerable fluctuations in the excitability of one or more nerve cells in the polysynaptic chain occurred.

The characteristics of the commonly observed repetitive discharge in sensory trigeminal neurones were similar to those observed by Amassian & de Vito (1957) in the cuneate nucleus. However, no late discharge comparable with that involving the relay in Clarke's column was observed. The long synaptic activation underlying the repetitive discharge probably resulted from temporal dispersion of presynaptic activity. Two synaptic pathways were probably available for synaptic activation of the neurone; one involved second-order neurones and their recurrent collateral axons, and the other the more direct monosynaptic pathway. At a low stimulus intensity synaptic discharge occurred via the converging recurrent collaterals, with relatively long latency. On increasing the stimulus intensity the neurone was fired by the more direct pathway, resulting in an abrupt shortening of the latency of discharge. The volley reaching the cell via the recurrent collateral now arrived while the cell was still partially refractory and only on activating more converging recurrent collaterals by increasing the peripheral stimulus did the cell discharge repetitively. Such a mechanism explains the observed quantal reduction in latency on increasing the stimulus intensity, the increases in the median threshold and the dispersion of the threshold distribution, and the recurrent peaks in the synaptic

potential which were recorded in some units. These observations cannot be readily explained in terms of long-lasting transmitter action (McIntyre, Mark & Steiner, 1956).

When the site of peripheral stimulation within the receptive field of a monosynaptically activated neurone was changed, only the presynaptic pathway was modified; alterations in the distribution of the threshold for excitation reflected this change in the presynaptic pathway. The activation of only a few converging fibres seems to be necessary to discharge a trigeminal neurone monosynaptically. If a large number of fibres converge on the nerve cell from the central part of the receptive field, excitation of the required few fibres would probably occur at a quite low stimulus intensity, because the density of fibres about the stimulating electrode would be high. If only a few fibres converge on the neurone from peripheral areas of the receptive field, the density of these fibres in the region of the electrode would be low, requiring both a higher average stimulus intensity to excite the required number of fibres (median threshold) and a greater change in stimulus strength to activate each additional fibre (dispersion of the threshold distribution). The same anatomical basis for the changes in threshold distributions within the receptive fields of polysynaptically activated nerve cells seems likely.

SUMMARY

1. The responses of neurones in the trigeminal main sensory nucleus to graded electrical stimulation of the upper lip of cat were investigated. A synchronous afferent volley evoked both an early (monosynaptic) and a later (presumably polysynaptic) discharge of neurones. In the latter group repetitive firing was common.

2. The distribution of the threshold for excitation of individual nerve cells was estimated and specified by two parameters, the median threshold and the variance of the distribution. The dispersion (estimated by the variance) of the threshold distribution of monosynaptically activated neurones was small, but for non-repetitively firing long-latency units it was up to four-hundredfold greater.

3. In the common type of repetitive discharge successive spikes had a progressively higher median threshold and an increasing dispersion of the threshold distribution.

4. For all neurones the latency was reduced on increasing stimulus strength. The latency of the first spike in labile repetitive discharges with increasing input decreased abruptly by an interval of 1–2 msec; the second spike in the train then developed at this interval after the first spike. It is suggested that temporal dispersion of the presynaptic volley involving the

recurrent collateral axons of neighbouring second-order neurones was the mechanism underlying the repetitive discharge.

5. The stimulus site within the receptive field modified the threshold distribution; towards the periphery of the field both the median threshold and the dispersion of the threshold distribution increased. This was thought to result from a decrease in the density of converging peripheral fibres at the edge of the receptive field.

This work was supported by a grant from the National Health and Medical Research Council, Australia. I wish to thank Professors P. O. Bishop and P. I. Korner for helpful criticism, and Miss Eva Spitzer for technical assistance.

REFERENCES

Amassian, V. E. & de Vito, J. L. (1957). La transmission dans le noyau de Burdach. In *Microphysiologie comparée des éléments excitables. Colloques Internationaux du Centre National de la Recherche Scientifique*, No. 67.

Åström, K.-E. (1952). On the central course of afferent fibres in the trigeminal, facial, glossopharyngeal and vagal nerves and their nuclei in the mouse. *Acta physiol. scand.* Suppl. 106, 209–320.

Bishop, P. O., Jeremy, D. & McLeod, J. G. (1953). Phenomenon of repetitive firing in lateral geniculate of cat. *J. Neurophysiol.* **16**, 437–447.

Cajal, S. R. (1909). *Histologie du système nerveux de l'homme et des vertébrés.* Vol. 1. Reprint 1953. Madrid.

Coombs, J. S., Eccles, J. C. & Fatt, P. (1955). Excitatory synaptic action in motoneurones. *J. Physiol.* **130**, 374–395.

Einarson, L. (1932). A method for progressive selective staining of Nissl and nuclear substances in nerve cells. *Amer. J. Path.* **8**, 295–307.

Erulkar, S. D., Rose, J. E. & Davies, P. W. (1956). Single unit activity in the auditory cortex of the cat. *Johns Hopk. Hosp. Bull.* **99**, 55–86.

Finney, F. J. (1952). *Probit Analysis*, 2nd ed. Cambridge University Press.

Freygang, W. H. (1958). An analysis of extracellular potentials from single neurons in the lateral geniculate nucleus of the cat. *J. gen. Physiol.* **41**, 543–564.

Glees, P. & Soler, J. (1951). Fibre content of the posterior column and synaptic connections of nucleus gracilis. *Z. Zellforsch.* **36**, 381–400.

Gray, J. A. B. & Malcolm, J. L. (1950). The initiation of nerve impulses by mesenteric Pacinian corpuscles. *Proc. Roy. Soc.* B, 96–114.

Hubel, D. H. (1957). Tungsten micro-electrode for recording from single units. *Science*, **125**, 549–550.

Hunt, C. C. (1955). Monosynaptic reflex response of spinal motoneurons to graded afferent stimulation. *J. gen. Physiol.* **38**, 813–852.

Kuffler, S. W. (1953). Discharge patterns and functional organisation of mammalian retina. *J. Neurophysiol.* **16**, 37–68.

Laporte, Y., Lundberg, A. & Oscarsson, O. (1956). Functional organisation of the dorsal spino-cerebellar tract in the cat II. Single fibre recording in the Fleschig's fasciculus on electrical stimulation of various peripheral nerves. *Acta physiol. scand.* **36**, 188–203.

Lindblom, U. F. (1958). Excitability and functional organisation within a peripheral tactile unit. *Acta physiol. scand.* **44**, Suppl. 153.

McIntyre, A. K., Mark, R. F. & Steiner, J. (1956). Multiple firing at central synapses. *Nature, Lond.*, **178**, 302–304.

Pecher, C. (1939). La fluctuation d'excitabilité de la fibre nerveuse. *Arch. int. Physiol.* **49**, 129–152.

Rose, J. E. & Mountcastle, V. B. (1954). Activity of single neurons in the tactile thalamic region of the cat in response to a transient peripheral stimulus. *Johns Hopk. Hosp. Bull.* **94**, 238–282.

SHERRINGTON, C. S. (1929). Some functional problems attached to convergence. *Proc. Roy. Soc.* B, **105**, 332–362.

SVAETICHIN, G. (1958). Component analysis of action potentials from single neurons. *Exp. Cell Res.* Suppl. 5, 234–261.

TASAKI, I., POLLEY, E. H. & ORREGO, F. (1954). Action potentials from individual elements in cat geniculate and striate cortex. *J. Neurophysiol.* **17**, 454–474.

TORVIK, A. (1957). The ascending fibres from the main trigeminal sensory nucleus. *Amer. J. Anat.* **100**, 1–13.

WINKLER, C. (1921). *Anatomie du Système Nerveux.* Tome 7, p. 82. Haarlem: Bohn.

J. Physiol. (1960), **153**, *pp.* 74–87
With 8 text-figures
Printed in Great Britain

CHARACTERISTICS OF RESPONSES FROM RECEPTORS FROM THE FLEXOR LONGUS DIGITORUM MUSCLE AND THE ADJOINING INTEROSSEOUS REGION OF THE CAT

BY C. C. HUNT AND A. K. McINTYRE
From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.

(*Received* 7 *March* 1960)

The flexor longus digitorum (FLD) in the cat is an unusual muscle in several respects. It consists of two separate heads, of which the lateral is the larger (the homologue of flexor longus hallucis in certain other species). The tendons of the two heads merge in the foot and the combined tendon splits into four subdivisions which are inserted into the terminal phalanges of the four digits (Reighard & Jennings, 1934). Thus the two heads of FLD have an identical action consisting of plantar flexion of the digits and protrusion of the claws. While this muscle behaves reflexly as an extensor, acting to oppose the force of gravity, it also has the unique function of claw protrusion. Possibly in relation to the latter function, it appears to be the only hind-limb muscle subject to long spinal inhibition from the forelimb (Lloyd & McIntyre, 1948). A recent study by Eccles, Eccles & Lundberg (1957) suggests that FLD might be atypical in still another regard, namely, in that volleys presumably confined to afferent fibres of the largest diameter range evoke unusually powerful reflex effects in a pattern of limb flexion. It was suggested that such action was produced by impulses in fibres from tendon organs. This flexor action has been considered to be significant in initiation of the flexor phase of stepping movements (Lundberg, 1959; Eccles & Lundberg, 1959).

Another point of interest in relation to FLD is that a small nerve from the region of the interosseous membrane courses between the medial and lateral heads of the muscle and merges with the nerve to FLD medial head. Since it seems likely that this interosseous nerve might frequently have been included with the FLD nerves in both anatomical and physiological studies, analysis of the receptor characteristics of its fibres is of obvious interest.

The present study had the twofold purpose of analysing the afferent-fibre diameter–receptor relation in the nerves to FLD, and of exploring the types of receptor served by fibres in the interosseous nerve. The findings indicate

that the diameter distributions of afferent fibres to muscle spindles and to tendon organs in the FLD nerves do not differ appreciably from those of gastrocnemius or soleus (Hunt, 1954), nor did there seem to be a significant difference in the relative proportion of muscle-spindle and tendon-organ receptors. Of perhaps greater interest was the finding of some hitherto undescribed receptors in the interosseous nerve. One of these is exceedingly sensitive to vibration and may well subserve vibration sensibility.

METHODS

Adult cats anaesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories) were used. After laminectomy and exposure of the lumbosacral region of the spinal cord, the tissues were covered with liquid paraffin equilibrated with 95 % O_2 + 5 % CO_2. The dura was then opened and dorsal and ventral roots L6–S2 were cut near the cord. The nerves to FLD medial head, FLD lateral head and the interosseous nerve were left intact and placed on separate pairs of stimulating electrodes. Other nerves in the hind limb and hip were cut.

Single fibres from the FLD and interosseous nerves were isolated in filaments of dorsal root. Conduction time was determined in each fibre from the interval between application of a stimulus (at twice threshold strength) and arrival of an impulse in the dorsal root filament. This time was measured by gating a 1 MC Berkeley counter for the duration of the interval. Simultaneous display of stimulus artifact and impulse on one beam and gating pulse on the other beam of a dual-beam oscilloscope (Tektronix model 502) insured that the proper interval was measured. On completion of the experiment the locations of stimulating cathodes and of proximal recording electrode were marked by ties of fine silk and the entire conducting path was excised and measured. From conduction times and distances the conduction velocities were calculated, a deduction of 0·1 msec being made for setting-up time. Diameters (μ) were estimated by dividing conduction velocity (in m/sec) by 6 (Hursh, 1939). In most experiments the tendon of the FLD muscle being studied was attached to a Statham strain gauge for isometric recording of tension. For observation of the discharge pattern of the muscle stretch receptors its output was displayed on one beam of the oscilloscope. Contraction of the muscle was produced by stimulation of a ventral root by single shocks or trains of stimuli. Body temperature was usually maintained between 37 and 39° C. Vibration at varying frequency was produced by a Goodmans (model V47) vibrator driven by a sine-wave oscillator through a transistor power amplifier.

RESULTS

A total of 275 afferent fibres from the FLD and interosseous nerves were isolated. Figure 1 represents a calculated diameter distribution of this entire sample. Comparison with the histological diameter distribution of Lloyd & Chang (1948) suggests that the present sample is deficient in fibres of the Group III range (1–4 μ) and perhaps excessive in proportion of Group I (12–22 μ) to Group II (4–12 μ) fibres. This is not surprising in view of the fact that detection of larger fibres is easier and that no special effort was made to isolate the smallest of the myelinated fibres. The distribution shown in Fig. 1 does indicate, however, that a reasonably comprehensive sample was obtained of fibres in the 5–22 μ range.

Afferent fibres in the FLD nerves

A total of 216 of the fibres isolated ran in the medial or lateral FLD nerves. Of these the most commonly encountered were fibres from muscle spindles (147 fibres); the next most frequent were tendon-organ fibres (53). Five of the fibres came from receptors usually associated with fibres of the interosseous nerve (see below). Also, two fibres were isolated which had receptors in the fascia or near the tendon. An additional nine fibres were isolated from FLD nerves which could not be activated by natural stimulation.

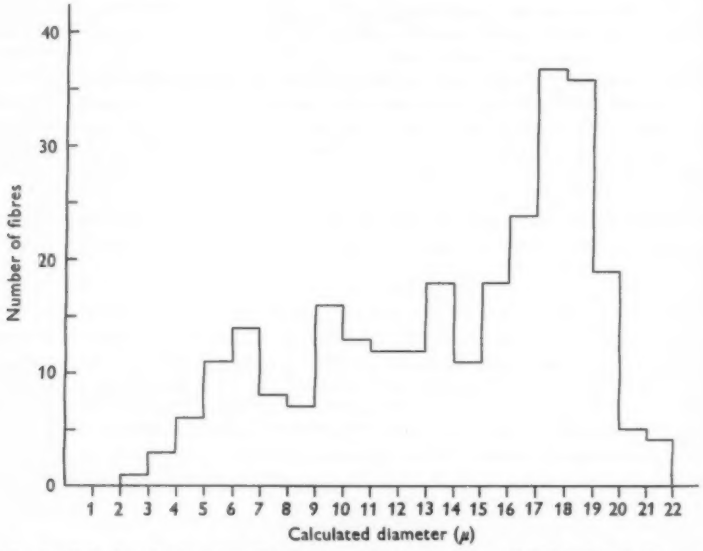


Fig. 1. Calculated diameter distribution of total sample of 275 isolated afferent fibres from FLD and interosseous nerves.

Afferent fibres from muscle spindles were identified by the discharge pattern during contraction indicating 'in parallel' behaviour (Matthews, 1933). A clear pause in discharge during the period of muscle shortening, particularly on ventral root stimulation and when some shortening was allowed, provided reasonably positive proof. Supplementary evidence was indicated by the usually low stretch threshold of muscle-spindle receptors and by the acceleration of discharge which occurred on raising the strength of tetanic stimulation of the ventral root sufficiently to excite fusimotor fibres. The diameter distribution of muscle-spindle afferent fibres is shown in Fig. 2. The distribution is clearly bimodal, with a minimum between the

modes occurring at 11–12 μ. The latter corresponds closely to that noted in the histological afferent-fibre spectrum of FLD nerves at 12–13 μ (Lloyd & Chang, 1948). The distribution of muscle-spindle afferent fibres in two modes, one in the Group I and the other in the Group II band, is similar to the spectrum of this type of fibre in soleus and in medial gastrocnemius (Hunt, 1954). The ratio of Group I : Group II muscle-spindle afferent fibres in the present study was about 1·6 : 1. This contrasts with the relatively greater proportion of Group II muscle-spindle afferent fibres found in soleus and medial gastrocnemius nerves (Hunt, 1954).

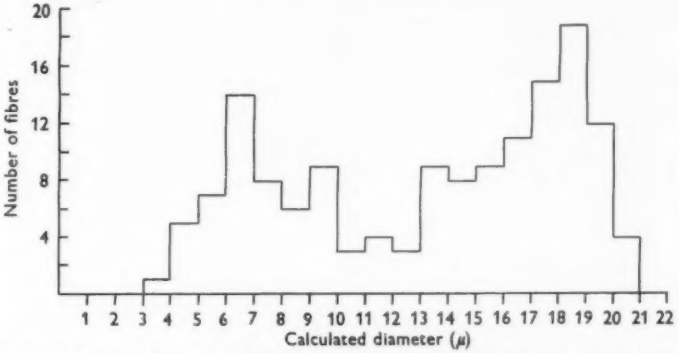


Fig. 2. Calculated diameter distribution of muscle-spindle afferent fibres, including two fibres in the interosseous branch.

Fibres whose discharge pattern showed 'in series' behaviour during muscle contraction, undergoing an increase in discharge during muscle shortening, were classed as coming from tendon organs. Receptors of such fibres generally had a high threshold to stretch; they were uninfluenced by fusimotor stimulation. The calculated diameter distribution is shown in Fig. 3. Although a few fibres lie in the Group II range, the distribution of diameters of tendon-organ fibres is essentially unimodal with most of the fibres in the 12–22 μ range and the peak at 17–18 μ. This agrees closely with the findings for soleus and medial gastrocnemius (Hunt, 1954) and confirms the earlier conclusion that the Group I band is functionally of dual nature, being comprised of fibres from muscle spindles (1*a*) and tendon organs (1*b*) (Kuffler, Hunt & Quilliam, 1951).

Two FLD fibres were from receptors of some interest. One, in the nerve to FLD lateral head, conducted at 75 m/sec. This receptor showed a spontaneous discharge synchronous with the heart beat. It could be made to discharge by slight pressure on or by lightly stroking the fascia over the muscle in a restricted region. On ventral root stimulation it fired briefly on both shortening and lengthening of the muscle. The other fibre, in the

nerve to the FLD medial head, conducted at 23·6 m/sec and discharged to strong pressure over the distal portion of the muscle and adjoining portion of tendon. It fired only briefly to strong stretch of the muscle but not during a twitch. Tetanic contraction evoked only a few impulses which continued for several seconds after cessation of stimulation.

Of the five fibres in the FLD nerves which had receptors of types usually found in the interosseous nerve, four were in the medial nerve and one in the lateral. Two of the fibres were connected to receptors of the type classified as tension receptors, while the remaining three were from another

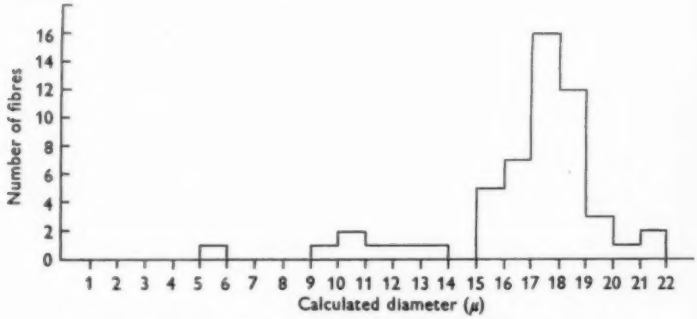


Fig. 3. Calculated diameter distribution of tendon-organ afferent fibres in FLD nerves.

variety designated tap receptors. The characteristics of these receptors are described below. It seems clear that the nerves to FLD, particularly to the medial head, may contain fibres some of which extend into the Group I range and are from other than muscle receptors. As will be discussed below, the interpretation of reflex effects from FLD nerves must take into consideration possible contamination from interosseous-type fibres.

Of the fibres isolated from the FLD nerves, nine could not be made to discharge by mechanical stimulation. This is an unexpectedly large number of muscle nerve fibres to be found unresponsive to the natural stimuli employed (see Hunt, 1954). Since a considerable number of fibres in the interosseous nerve could not be made to discharge, and since fibres of interosseous nerve type do run in the FLD nerves, the unresponsive units may well have come from receptors outside the muscle.

Fibres in the interosseous nerve

A total of sixty-one fibres were isolated from this nerve, of which forty-eight could be activated by the available means of mechanical stimulation. The behaviour to various types of stimuli permitted a separation of the

receptors of such fibres into three major categories which will be described separately.

1. *Vibration receptors.* These units were so designated because of their unique sensitivity to vibratory stimuli. Although these receptors differed in threshold, most of them were extraordinarily sensitive to vibration, particularly when transmitted via bone. The preparation was mounted on

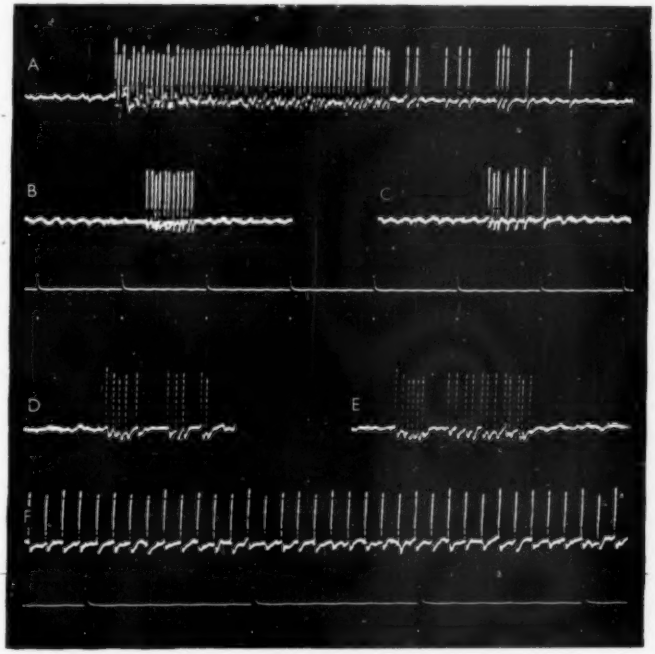


Fig. 4. Responses recorded in a fibre from a vibration receptor. A, to tap of metal upright; B, to dropping box of paper matches on heavy base plate; C, to tapping foot on floor; D and E, to light stroking of skin over pretibial region; F, to application of tuning fork vibrating at 100 c/s to metal upright. Time marker, 100 msec; time marker above pertains to A, B and C, time marker below to D, E and F.

a heavy aluminium base plate by means of steel pins inserted into the lower ends of the femur and tibia, as well as by clamps on the ilia and spinous processes above the laminectomy. A brief tap causing vibration of one of the metal uprights attached to this base elicited a discharge from vibration receptors which appeared to follow the frequency of vibration (Fig. 4A). Some were so sensitive that slight jarring of the floor some distance away (Fig. 4C) or the vibration produced by dropping a small package of paper matches on the heavy base plate (Fig. 4B) was sufficient to evoke discharge.

Another very effective stimulus was light stroking of the skin over the pretibial region by a wisp of cotton or by a soft brush (Fig. 4D, E). The most effective stimulus to vibration receptors was provided by application of a tuning fork over the bony structures in the lower leg. Figure 4F shows the response of a vibration unit to a tuning fork vibrating at 100 c/s applied to an upright attached to the base plate. In this case the receptor discharged one impulse to each cycle. While vibration receptors were extremely sensitive to transient mechanical stimuli, they failed to respond to sustained pressure or to squeezing of bony structures in the lower leg. These receptors invariably appeared to be rapidly-adapting.

Further investigation of the response of vibration receptors was undertaken by using an electromagnetic vibrator actuated by a variable-frequency sine-wave oscillator. The vibrating element was attached to a steel pin which was pressed against the lateral malleolus (the connexion between the tibia and base plate being loosened). Although the amplitude of vibration diminished considerably above 100 c/s, owing to the characteristics of the vibrator used, it was found that vibration receptors often followed frequencies up to 700 c/s. It is possible that the upper frequency limit would be even higher in response to a larger amplitude of vibration. Most vibration receptors failed to discharge at frequencies of vibration below 40 c/s. The response of an illustrative unit to vibration at varying frequency is shown in Fig. 5. Over a considerable range the receptor discharge followed the frequency of vibration in a 1:1 ratio. However, in the range 130–160 c/s two impulses were evoked per vibration cycle. At frequencies greater than 400–500 c/s this receptor sometimes fired on alternate cycles of vibration. These findings were generally representative of the vibration receptors examined, although the exact frequencies at which the receptor followed the vibration frequency on a 1:1, 2:1 or 1:2 basis varied from one unit to another. Also, other ratios of discharge to vibratory cycle were occasionally noted. For example, one unit showed a ratio of 3:1 over a limited range of frequency.

As might be expected from their very low threshold to transient mechanical stimuli, vibration receptors also discharged during contraction or stretch of nearby muscles. Figure 6B shows the response of such a unit to contraction of the lateral FLD muscle. As was frequently observed, a series of impulses was discharged during the muscle twitch.

2. *Tap receptors.* Another category of receptor frequently encountered was one which responded to brief mechanical stimuli applied to bony structures in the leg or foot. These receptors were rapidly-adapting. The most effective stimulus was a sharp tap with a finger or blunt instrument on the tibia (Fig. 7B). These receptors were much less sensitive to mechanical stimuli than the vibration receptors and failed to respond to a vibrating

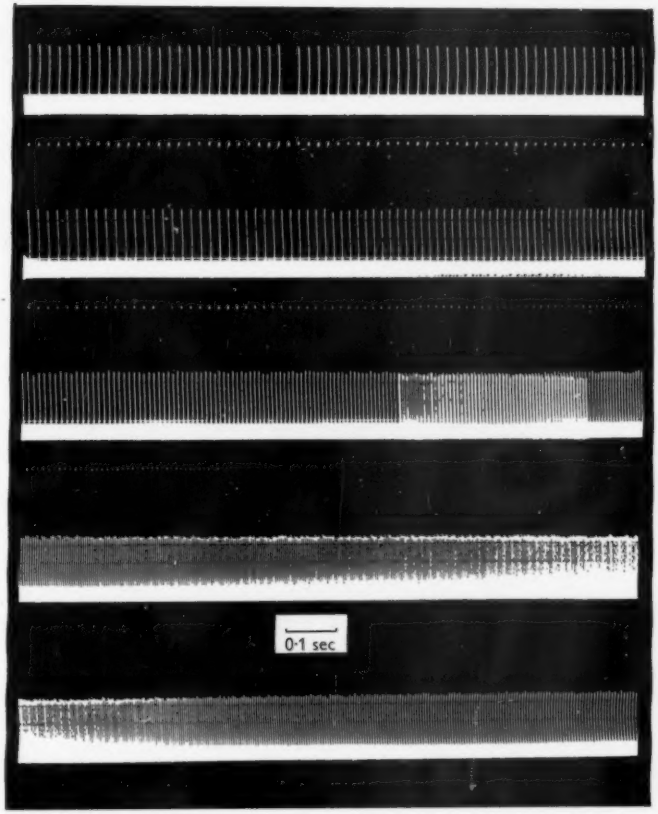


Fig. 5. Response of a vibration receptor to vibrating stimulus at varying frequency. Frequency gradually increased for first four records to a maximum of about 400 c/s, then decreased in lowest record. Sine wave below impulse record indicates driving frequency. Receptor follows frequency of vibration except for range 130–160/sec, where there are 2 impulses/vibration. Time marker, 0·1 sec.

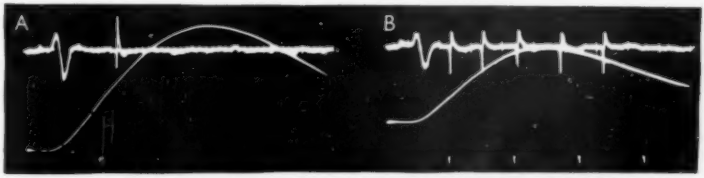


Fig. 6. Responses of a tension receptor (A) and of a vibration receptor (B), both in the interosseous region, during contraction of the lateral FLD muscle. Lower traces show isometric tension record. Time marker, 10 msec. Slow potential in upper traces is artifact from muscle action potential.

tuning fork held against the bone. It is possible that the tap and vibration receptors are similar in nature but have thresholds that are widely different. The fact that the afferent fibres from the vibration and tap receptors have the same range of diameter, as described below, could be taken to support this suggestion.

3. *Tension receptors.* These were activated by mechanical stimuli which must have caused tension upon, or deformation of, the interosseous membrane or associated structures. Squeezing the tibia and fibula together or pressure through the pretibial muscles were among the most effective

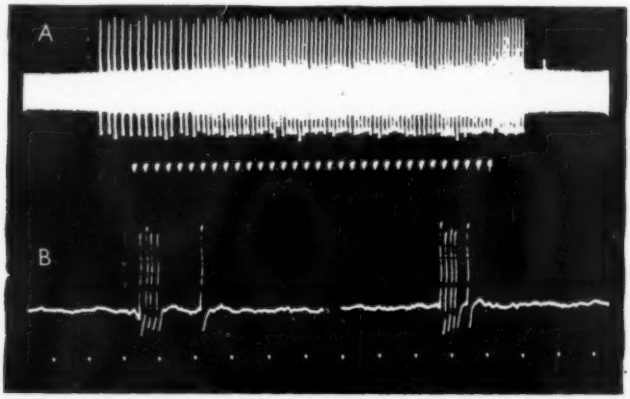


Fig. 7. Responses of a tension receptor (A) and a tap receptor (B), both in the interosseous region. A, effect of squeezing tibia and fibula together on tension unit. B, left and right records indicate responses of tap receptor to brief tap of finger on tibia. Time marker, 100 msec.

means of exciting these receptors. A typical response, illustrated in Fig. 7A, shows the discharge in response to squeezing the tibia and fibula together. A sustained discharge resulted for the duration of the stimulus. Another effective means of eliciting discharge from tension receptors was by stretching the FLD muscles, particularly the lateral head. Moderate stretch often elicited a sustained discharge, and in a few instances strong stretch of the FLD lateral head was the only effective way of evoking discharge from these receptors. The responses of tension receptors to muscle stretch bear some similarity to those of tendon organs. However, differentiation was no problem, for the responses during muscle contraction differed considerably. While tension receptors often showed one or a few impulses during contraction of one or both heads of FLD (Fig. 6A), they never showed the high-frequency discharge during muscle shortening which characterizes tendon organs. Tension receptors also could be caused to

discharge by manoeuvres which failed to excite tendon organs, such as squeezing the tibia and fibula together.

Tension receptors were always found to be slowly-adapting, maintaining discharge as long as an effective stimulus was applied. Lack of exposure in preparations used in the present study prevented evaluation of responses to phasic stimuli. However, tension receptors failed to respond to brief mechanical stimuli transmitted through the bones of the lower limb. For example, tapping the tibia or fibula or application of a vibratory stimulus to these regions did not evoke discharge.

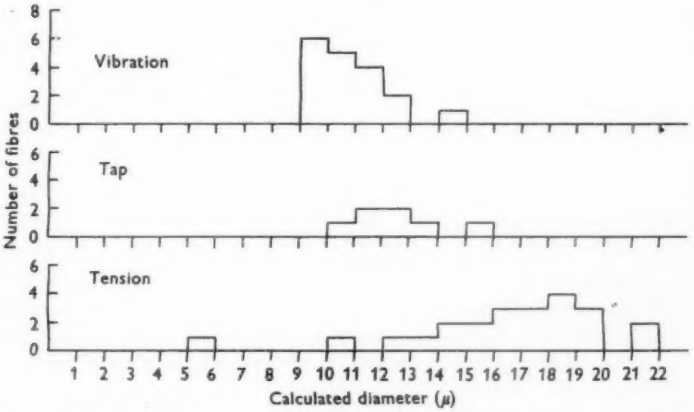


Fig. 8. Calculated diameter distributions of afferent fibres from vibration, tap, and tension receptors. All fibres were in the interosseous nerve except five, which were in FLD nerves.

The diameter distributions of interosseous-type afferent fibres associated with vibration, tap, and tension receptors are shown in Fig. 8. Included in this distribution are the five units from interosseous-type receptors which were in the FLD nerves, three of which were tap-type receptors and two of the tension type. The diameter distributions of vibration and tap units were similar and confined to a range of calculated diameters of 9–16 μ. In contrast, the fibres from tension receptors extended over a considerably larger range of diameter, the majority of fibres occupying a band comparable in size to Group I of the muscle afferent spectrum. Since fibres of tension receptors were found in the FLD nerves and since the interosseous nerve may easily be included with the FLD nerves when these are used as conditioning sources for reflex studies, interpretation of the effects of Group I volleys should take into account actions produced by tension receptor fibres.

In addition to the fibres with receptors of the vibration, tap, or tension type, two fibres from the interosseous nerve (which must have taken an aberrant course) were isolated which showed discharge patterns typical of muscle spindles located in the lateral head of FLD. Also, thirteen other fibres of the interosseous nerve were isolated for which no effective natural stimulus could be found. Three of these were slowly-conducting (with velocities under 33 m/sec) while ten had conduction velocities between 50 and 96 m/sec. In this regard it is important to note that the exposure of tissues innervated by the interosseous nerve was limited in the present experiments and it may well have been impossible adequately to stimulate endings of high threshold. Preliminary experiments made a number of years ago by one of us (A. K. McI.) in collaboration with J. C. Eccles did indicate the presence of afferent fibres from the region of the ankle and proximal tarsal joints, with receptors of high mechanical threshold. As noted above, the relatively large number of fibres in the FLD nerves which could not be identified as to receptor function might have a similar explanation.

DISCUSSION

The results of the present analysis of afferent fibres from stretch receptors of the FLD muscles of the cat indicate that there is nothing unusual about the diameter distributions of fibres belonging to the different receptor types nor about the relative numbers of spindle and tendon-organ endings. As is the case in soleus and medial gastrocnemius (Hunt, 1954), spindle afferent fibres fall into two groups corresponding to the Group I and Group II bands of the histological afferent fibre spectrum and presumably representing the afferent pathways from primary and secondary endings respectively. Tendon-organ fibres, on the other hand, are essentially confined to the Group I band and their diameters are very nearly the same as those of the Group I spindle afferent fibres. There is no indication of an unusually large proportion of tendon-organ fibres in the FLD muscles which might be suggested by the exceptionally powerful reflex actions attributed to volleys in Group Ib fibres (Eccles *et al.* 1957).

There appears to be little prospect that Ia fibres could be stimulated by weak electrical shocks to the partial or complete exclusion of stimulation of Ib fibres in the FLD nerves, as has been claimed for certain other hind-limb muscle nerves (Bradley & Eccles, 1953; Eccles *et al.* 1957). In this respect FLD afferent fibres resemble those of the triceps surae (Hunt, 1954), in which the diameter distributions of Ia and Ib fibres show almost complete overlap.

The unusual feature about the FLD nerves is not the relation between muscle stretch receptors and the diameter distribution of their fibres but

the close association of the interosseous nerve with the muscle nerves proper. Apart from the physical proximity and the merging of this nerve with the medial branch to the FLD at varying distances from the muscle, some afferent fibres serving receptors of the interosseous types have been found in the muscle nerves themselves. In view of the fact that some of these aberrant interosseous fibres are of large diameter, occupying the Group I range, it appears that it would be impossible to evoke by electrical stimulation volleys in either FLD nerve that could with confidence be regarded as engaging only afferent fibres from muscle receptors. This would be the case even if adequate separation of the interosseous nerve from the FLD muscle nerves were achieved. Inclusion of this branch with the muscle nerves, or inadequate precautions to prevent cross-stimulation at the point where the interosseous branch joins the medial nerve to the FLD, would lead to a much larger contamination of the muscle afferent volley with impulses in interosseous fibres, many of which conduct at Group I velocity. The likelihood of contamination of FLD volleys by impulses in fibres from interosseous-type receptors should be considered in interpreting the central actions produced by such volleys, since it is not yet known whether the reflex effects produced by activity in interosseous-type receptors are similar to those evoked by tendon-organ activity.

The fibres from interosseous tension receptors may be as large in diameter as the largest Group I fibres in muscle nerves and may conduct at velocities in excess of 120 m/sec. Although it is now apparent that fibres in cutaneous nerves may conduct as rapidly as 90–100 m/sec, fibres conducting at the upper limit of the Group I range have previously been considered to come exclusively from muscle stretch receptors. However, it seems clear that the tension receptors differ from muscle spindles and tendon organs and are probably located in the interosseous membrane, periosteum or related structures rather than in muscle. Hence, fibres of the entire range of the Group I diameter band can no longer be considered as arising solely from muscle receptors.

The most interesting of the receptors connected with fibres in the interosseous nerve are those sensitive to vibration. It seems very likely that these receptors must be of the same type as those responsible for the cortical responses to very slight mechanical stimuli described by Mountcastle, Covian & Harrison (1952) and by Mountcastle & Powell (1959). The extreme sensitivity of the lowest threshold vibration receptors is most striking. In fact, one such unit responded to nearby loud sound, suggesting their sensitivity might even approach the range of sensitivity of cochlear receptors. The possibility seems very likely that the vibration receptors are concerned specifically with reception of vibratory stimuli and must be concerned with the conscious perception of vibration. Although

other receptors, such as the muscle spindle (Kuffler *et al.* 1951), may follow vibratory stimuli over a limited range, the vibration receptors described in the present study follow a wide range of frequencies and appear to have a much greater sensitivity to this form of stimulation than any other known receptors in skeletal structures. The preliminary observation made by one of us (A. K. McI.), that electrical stimulation of the interosseous nerve readily evokes potentials in the sensory receiving zones of the cerebral cortex, is consistent with the view that such receptors may be involved in vibration perception.

Although the vibration receptors appear to be optimally sensitive to the application of vibratory stimuli to the bony prominences, they also respond to vibrations transmitted through the foot pad and they may be expected to signal to the animal slight vibratory movements of the supporting surface, almost in the manner of a seismograph. Conceivably such a sensing system could be of importance in detection of prey. It would be of considerable interest to determine how widespread is the distribution of this type of very sensitive vibration receptor in regions other than the hind limb and in species other than the cat. The location and morphology of the vibration receptors also remain to be found.

The possible relation between vibration receptors and the less sensitive tap receptors is still to be determined. Also, the rather large number of interosseous fibres unidentified as to receptor type suggests the need for further experiments, in which more adequate exposure permits the application of high-intensity mechanical stimuli to localized areas in the region innervated by the interosseous nerve.

SUMMARY

1. An analysis of receptor characteristics and fibre diameter of 275 afferent fibres from the nerves to flexor digitorum longus (FLD) muscle and the associated interosseous nerve has revealed the following:

2. Fibres from muscle spindles in the FLD muscles have a bimodal distribution of diameter, the modes occurring in the Group I and II bands. Fibres from tendon organs showed a unimodal distribution essentially confined to Group I.

3. Several types of receptor, whose physiological activity has not previously been described, have been found associated with interosseous nerve fibres: (*a*) tension receptors, which have fibres extending to the maximal size of the Group I band and show a slowly-adapting discharge to pull or tension on structures of the interosseous region—their location has not yet been established; (*b*) vibration receptors, which respond to vibratory stimuli between 40 and 700/sec, are rapidly-adapting and often extremely sensitive—they are probably concerned with vibration sensibility; (*c*) tap

receptors, possibly a high-threshold variant of *b*, which respond to brief tap of bony structures around the ankle and are rapidly-adapting.

4. Some fibres with interosseous-type receptors have been found in the FLD nerves and conversely a few fibres from muscle spindles have been noted to course in the interosseous nerve. FLD nerves cannot be regarded as purely muscle nerves and the reflex effects of volleys in these nerves may depend, in part, on actions evoked by fibres from interosseous-type receptors.

This work was supported by a research grant (B–1320) from the National Institutes of Health.

REFERENCES

BRADLEY, K. & ECCLES, J. C. (1953). Analyses of the fast afferent impulses from thigh muscles. *J. Physiol.* **122**, 462–473.

ECCLES, J. C., ECCLES, R. M. & LUNDBERG, A. (1957). Synaptic actions on motoneurones caused by impulses in Golgi tendon organ afferents. *J. Physiol.* **138**, 227–252.

ECCLES, R. M. & LUNDBERG, A. (1959). Supraspinal control of interneurones mediating spinal reflexes. *J. Physiol.* **147**, 565–584.

HUNT, C. C. (1954). Relation of function to diameter in afferent fibers of muscle nerves. *J. gen. Physiol.* **38**, 117–131.

HURSH, J. B. (1939). Conduction velocity and diameter of nerve fibers. *Amer. J. Physiol.* **127**, 131–139.

KUFFLER, S. W., HUNT, C. C. & QUILLIAM, J. P. (1951). Function of medullated small-nerve fibers in mammalian ventral roots: efferent muscle spindle innervation. *J. Neurophysiol.* **14**, 29–54.

LLOYD, D. P. C. & CHANG, H. T. (1948). Afferent fibers in muscle nerves. *J. Neurophysiol.* **11**, 199–208.

LLOYD, D. P. C. & MCINTYRE, A. K. (1948). Analysis of forelimb-hindlimb reflex activity in acutely decapitate cats. *J. Neurophysiol.* **11**, 455–470.

LUNDBERG, A. (1959). Integrative significance of patterns of connections made by muscle afferents in the spinal cord. *XXI Congr. int. cienc. fisiolog.*, Buenos Aires, pp. 100–105.

MATTHEWS, B. H. C. (1933). Nerve endings in mammalian muscle. *J. Physiol.* **78**, 1–33.

MOUNTCASTLE, V. B., COVIAN, M. R. & HARRISON, C. R. (1952). The central representation of some forms of deep sensibility. *Res. Publ. Ass. nerv. ment. Dis.* **30**, 339–370.

MOUNTCASTLE, V. B. & POWELL, T. P. S. (1959). Central nervous mechanisms subserving position sense and kinesthesis. *Johns Hopk. Hosp. Bull.* **105**, 173–200.

REIGHARD, J. & JENNINGS, H. S. (1934). *Anatomy of the Cat.* New York: Henry Holt and Co.

J. Physiol. (1960), **153**, *pp.* 88–98
With 8 text-figures
Printed in Great Britain

PROPERTIES OF CUTANEOUS TOUCH RECEPTORS IN CAT

BY C. C. HUNT AND A. K. McINTYRE*
From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.

(*Received* 14 *March* 1960)

In their analysis of discharge patterns in cutaneous afferent fibres in cat, Maruhashi, Mizuguchi & Tasaki (1952) noted some fibres which showed discharge to mechanical stimulation of small receptive fields, often with spots of high sensitivity. The receptors adapted rapidly. Recently Witt & Hensel (1959) described responses in cutaneous fibres of cat with receptors sensitive to both mechanical and thermal stimuli. The receptors of such fibres often displayed localized areas of mechanical sensitivity. They also showed many of the characteristics of cold receptors previously described in the tongue (Hensel & Zotterman, 1951*a*), responding particularly to phasic cooling.

In the course of a study of fibre diameter and receptor function in isolated fibres of sural nerve, described in the paper which follows (Hunt & McIntyre, 1960), it was found that a type of receptor could be categorized which possessed features some of which were noted by Maruhashi *et al.* (1952) and by Witt & Hensel (1959). These receptors, which, together with their fibres, have been called touch units, have highly localized regions of mechanical sensitivity and many show responses to thermal as well as tactile stimuli. This paper is concerned with a detailed description of touch receptors in the skin of cat innervated by the sural nerve. The data are derived from study of 114 isolated touch units.

METHODS

Adult cats anaesthetized with pentobarbital sodium (Nembutal; Abbott Laboratories) were used. After laminectomy in the lumbosacral region, the exposed spinal cord and roots were covered with paraffin oil equilibrated with 95 % O_2 and 5 % CO_2. Dorsal and ventral roots from L 6 to S 2 were divided near the cord. By an incision over the popliteal fossa the sural nerve was exposed and placed intact on stimulating electrodes in a pool of paraffin oil. Other nerves in the hind limb and hip were cut. Single afferent fibres were isolated in filaments of dorsal root.

Skin temperature was measured by a small bead thermistor introduced subcutaneously at some distance from the receptor under study and then passed under the skin until it rested a few millimetres from the receptor, pointing outwards against the skin. In this position it responded within a few seconds to changes in skin temperature produced by

* Present address: Department of Physiology, University of Otago, Dunedin, New Zealand.

cooling with ice or ether or by warming by infra-red radiation. While the thermistor temperature cannot be taken as indicating directly the temperature of the receptor, it served as a reasonable approximation to the latter. The thermistor was connected to a bridge circuit, the output of which could be read on a calibrated meter or displayed on one beam of the cathode-ray oscilloscope. A switching arrangement permitted resistance equal to the thermistor resistance at known temperatures to be connected to the bridge in place of the thermistor, thus providing reference calibration during the course of the experiment.

Mechanical stimulation of the skin was produced by pressure against the skin by a blunt stylus attached to the piston of a syringe. Air pressure was applied to the syringe through a variable-demand valve regulator, the duration of the constant pressure being controlled by a transistor-regulated relay.

RESULTS

Responses to mechanical stimuli

Touch receptors could usually be readily distinguished from other types of receptors in the skin that were sensitive to mechanical stimulation. The latter, which are more fully described in the paper which follows, include hair receptors, pressure receptors, and probable nociceptive units. Hair receptors responded to movement of hairs without otherwise deforming the skin and usually displacement of any of a considerable number of hairs sufficed to evoke discharge. Touch receptors, on the other hand, usually discharged only when the skin was actually touched and careful mapping indicated that the receptive area was limited to one or more very discrete spots. Further, hair receptors were invariably rapidly-adapting, while touch receptors were generally slowly-adapting. Occasionally very sensitive touch receptors discharged to movement of hairs, but they could be distinguished from hair receptors by other aspects of their behaviour. Pressure receptors were of much higher threshold and had no sharply localized receptive spots.

Touching the skin with a large blunt object evoked discharge in fibres or touch receptors which was usually sustained for the duration of the stimulus. In response to this type of stimulus the discharge was of moderate frequency (up to about 100/sec) and was graded by the degree of pressure employed. Generally only light touch was required to initiate discharge. Often traction on the skin at some distance from the receptive area would evoke a low-frequency discharge from touch receptors, but they were always much less responsive to such remote stimulation than highly sensitive hair receptors (Hunt & McIntyre, 1960).

More detailed examination of the mechanical responses of touch receptors was carried out with a fine probe such as a cat's vibrissa. To such a mechanical stimulus the receptive region was found to be limited to very discrete spots, numbering one to five in a given touch unit. In units showing multiple receptive spots these were usually arranged in a cluster, the individual spots being 1–5 mm apart. Some of the arrangements

observed are shown in Fig. 1. The number of receptive spots per unit observed in 77 touch fibres was as follows: 1 spot—30 fibres, 2 spots—34 fibres, 3 spots—8 fibres, 4 spots—2 fibres and 5 spots—3 fibres. Light touch to regions between sensitive spots usually caused no discharge, although sufficient pressure to such regions could do so, presumably by the deformation transmitted to a receptive spot. Only a few units were found in which the spots were not highly localized. In these, light touch to an area of some 30–60 mm² was capable of producing response, but even in such units several spots of heightened sensitivity were noted.

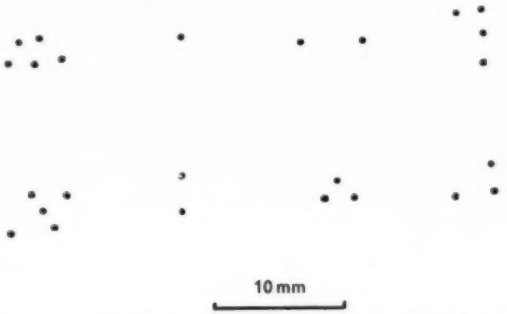


Fig. 1. Examples of the distribution of receptive spots taken from eight touch units.

The discharge evoked by mechanical stimulation of a discrete receptive spot could be graded in frequency by intensity of stimulation but could be of much higher frequency than that evoked by more diffuse pressure. Indeed, touch of a discrete receptive spot by localized pressure of moderate intensity characteristically produced a high-frequency train of impulses (up to about 400/sec) which tended to fall in frequency with maintained stimulation, although discharge usually continued until stimulation ceased (Figs. 2, 7). In contrast, the discharge to more diffuse pressure, while showing a lower initial frequency, tended to fall off less in frequency with maintained stimulation.

Repeated probing of a receptive spot sometimes led to unresponsiveness, although if a fibre had multiple receptive spots the remaining spots continued to respond to mechanical stimuli. The onset of block was associated with a diminution in number and frequency of impulses evoked by a tactile stimulus of a given duration and intensity to the receptive spot (Fig. 2). Together with this reduction in response there occurred an increase in rate of adaptation, so that impulses were evoked only at the onset of the tactile stimulus. Finally, there was absence of discharge to the mechanical stimulus even if this were made several times more intense than that

which originally evoked a high-frequency discharge. Following a period of rest of 5–15 min some blocked receptive spots recovered their responsiveness. Heavy pressure or pinching the skin, as was sometimes employed in seeking responses of possible nociceptive units, seemed to predispose toward block of touch-receptive spots.

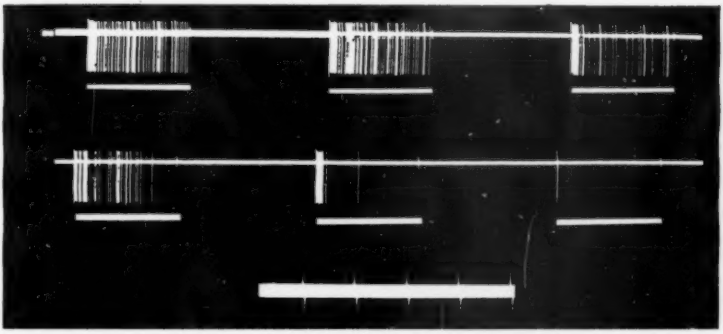


Fig. 2. Responses elicited from a touch unit by successive stimuli to a receptive spot, showing the development of block. Thermistor temperature 35° C. Horizontal bars indicate approximate duration of mechanical stimulus. Time marker, 1 sec.

Responses to temperature

A large number of the touch units examined displayed background discharge at the usual resting levels of skin temperature (32–35° C). At a constant temperature the discharge was often aperiodic, particularly when the average frequency was low. Changing the skin temperature to a new steady level usually altered the average discharge frequency, as is shown by the example in Fig. 3. This fibre showed discharge to tactile stimulation of discrete receptive spots, was slowly-adapting and displayed the usual characteristics of touch units. The background discharge in the absence of tactile stimulation is shown at three fairly constant levels of skin temperature (Fig. 3). This discharge had its highest average frequency at a thermistor temperature of 30° C; when the temperature was raised to 34·5 or lowered to 25·6° C the average frequency fell. When warmed or cooled sufficiently the discharge ceased. Figure 4 illustrates four examples of the relation between average discharge frequency and static temperature level as recorded by the thermistor. Units varied considerably as to the temperature at which peak frequency of discharge occurred, as well as to the range of temperature associated with discharge. In general the temperature range associated with discharge was approximately 22–42° C, although in many units the range was more narrow. The curves of Fig. 4

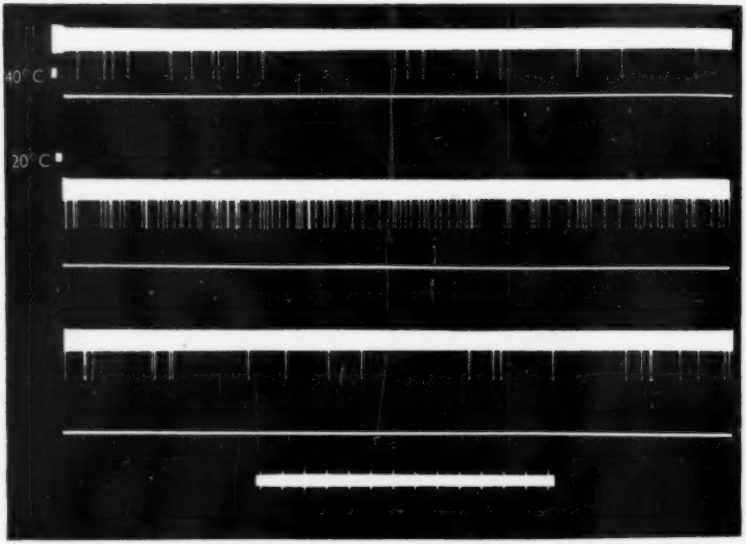


Fig. 3. Base-line discharge in a touch unit at three different skin temperatures. Thermistor temperature at onset of upper record 34·5, middle record 30, lower record 25·6° C. Time marker, 1 sec.

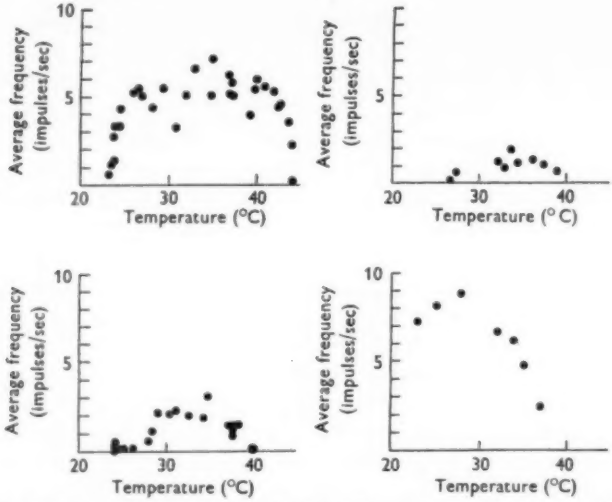


Fig. 4. Relation between base-line discharge frequency and skin temperature in four touch units. Ordinate, average frequency (impulses/sec). Abscissa—thermistor temperature.

are selected from units showing the most reproducible frequency changes at various temperature levels. In this selected group considerable variability of mean frequency is evident at the various temperature levels. In many other units such variation was more evident and it was difficult to obtain reproducible curves relating average frequency to temperature (cf. Witt & Hensel, 1959).

In addition to units which showed discharge with a frequency maximum in the 28–38° C range, others were found which showed no discharge until the temperature as recorded reached 40–42° C, whereupon repetitive discharge began and as the temperature was further raised the discharge frequency increased until it abruptly ceased. The upper limit of temperature studied was limited to about 45° C, in order to avoid tissue damage. On removal of the heat source, rapid cooling from the temperature maximum was generally accompanied by a discharge at considerably higher

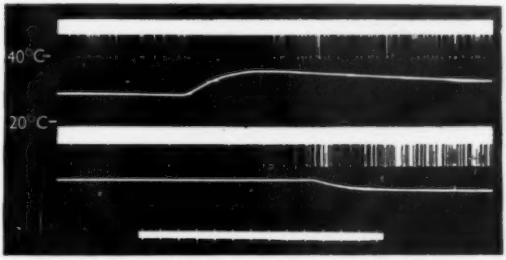


Fig. 5. Effect of warming and cooling on base-line discharge of a touch unit. Upper record, application of warm water raised temperature from 29 to 36° C. Lower record, application of cold water lowered temperature from 36 to 32·5° C. Time marker, 1 sec.

frequency than during warming. Indeed, a number of units were observed which showed no discharge to warming and discharged only after the heat source was removed.

An example of a unit which exhibited responses similar to cold receptor units (Hensel & Zotterman, 1951*a*; Witt & Hensel, 1959) is shown in Fig. 5. Allowing cold water to flow over the receptive area caused a marked increase in discharge. In contrast, similar application of warm water produced an inhibition of the base-line discharge during the period of phasic warming. There seemed to be no apparent mechanical stimulation associated with the latter. Direction and rate of temperature change are important determinants of responses of touch units to thermal stimuli. The most effective stimulus was usually rapid cooling and some touch units responded only during sudden phasic lowering of temperature.

Not all units behaved in a manner analogous to the responses of cold

receptors. The unit of Fig. 6 is an example of such an exception. As temperature was raised no discharge occurred until the thermistor temperature was above 41·2° C. With further temperature increase the discharge continued until it stopped abruptly at about 43·5° C. Removal of the infra-red heat source was followed by a fall in temperature at a rate equivalent to that which occurred during warming, but no discharge was evoked. At least over the temperature range shown, this unit was more responsive to warming than cooling. However, rapid cooling was quite effective in evoking discharge, as can be seen in the lower record of Fig. 6, which illustrates the response to application of ether to the skin.

The findings described above suggest that many touch receptors have some properties of cold receptors. Witt & Hensel (1959) have previously noted receptors of cat skin which behaved in several respects like cold receptors and which also responded to mechanical stimuli (see Discussion).

Fig. 6. Effect of slow warming and cooling and of rapid cooling on a touch unit. Upper record shows gradual warming from 41·2 to 43·6° C and subsequent cooling when infra-red heat was removed, the temperature fall being from 43·6 to 41° C. Lower record shows discharge evoked by rapid cooling from 40·6 to 20° C.

Effects of temperature on responses to tactile stimulation

With a constant-pressure stimulus which could be delivered for a predetermined duration (see Methods), the responses of touch units to mechanical stimulation were studied at varying skin temperatures. It was found that responses to constant tactile stimuli varied as a function of temperature. At the extremes of the temperature range studied touch receptors often completely failed to respond to the mechanical stimulus. This failure of response commonly occurred below 22 and above 42° C. In the range between these extremes the discharge to a constant mechanical stimulus was significantly altered by skin temperature. The unit of Fig. 7 displays some of the effects of cooling. The uppermost record (*A*) shows a base-line discharge and the increase in discharge which accompanied cooling. Following this, two applications of a constant-pressure stimulus (2 sec duration) to a receptive spot caused a sustained discharge during

the period of stimulation. The second record (*B*) shows a period of further cooling which was accompanied by discharge and the responses to subsequent mechanical stimulation. Although the temperature had fallen from about 32–24·7° C, the responses to mechanical stimuli were approximately similar. The third (*C*) and fourth (*D*) records show the effects of still further cooling: during the cooling discharge again occurred. At a temperature of 20° C two applications of the pressure stimulus of 2 sec duration resulted only in 3 or 4 impulses at the onset of the stimulus. A similar effect occurred at the upper temperature extreme studied (about 43° C.)

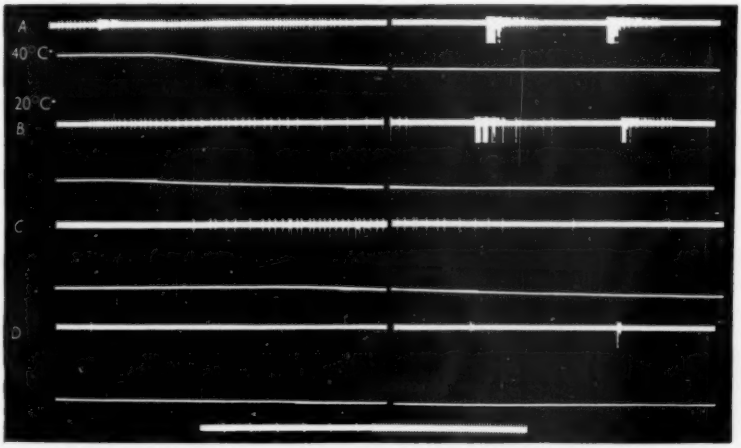


Fig. 7. Effect of cooling on responses of a touch receptor to mechanical stimulation. Upper record (*A*) shows cooling from initial temperature of 38·2 to 32·4° C; at right responses to two pressure pulses (2 sec duration) applied to receptive spot (at average temperature of 31·8° C). Second record (*B*) shows further cooling from 28·8 to 24·7° C; at right responses to mechanical stimulation at temperature 24·7° C. Third (*C*) and fourth (*D*) records show still further cooling from 24·7 to 20° C; at right of lowest record are responses to two pressure pulses at temperature 20° C. Note brief responses although duration of pulse was similar to that in right upper record. Time marker, 1 sec and 0·1 sec.

If the intensity of mechanical stimulus in Fig. 7 had been constant for its duration, the brief discharge at 20° C would indicate that the receptor adapted more rapidly at this temperature than at 31·8 and 24·7° C. Another possible explanation of these results would be that the mechanical deformation was greater at the onset of the pressure pulse and that the change in discharge following cooling resulted from an increased threshold of the receptor. However, strong pressure by a finger to the receptive field, which at normal temperatures evoked sustained discharge, elicited only

a brief discharge on application when the skin was cooled or warmed. These findings indicate that although touch receptors generally adapt slowly over a considerable temperature range, they can adapt rapidly when the skin is sufficiently warmed or cooled.

Certain effects of tactile stimulation on the base-line discharge of touch units may also be seen in Fig. 8. The base-line discharge was interrupted following the application of a mechanical stimulus, the response to the pressure pulse being followed by a period of silence. In some circumstances the base-line discharge reappeared after a short interval, but repetition of the response to mechanical stimulation led to a longer silence of the base-line discharge. A similar period of cessation of background discharge was seen after high frequency tetanic stimulation of the sural nerve.

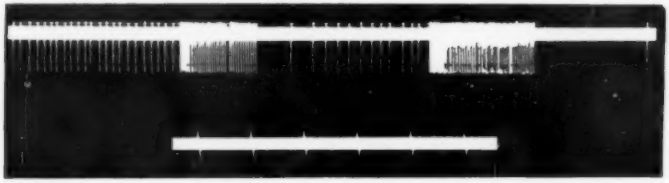


Fig. 8. Response of touch unit showing base-line discharge and responses to two pressure pulses. Note brief pause in base-line discharge after first mechanical stimulus and the more prolonged pause after the second. Thermistor temperature 35·6° C. Time marker, 1 and 0·1 sec.

DISCUSSION

Touch receptors have been shown to signal tactile stimulation of the skin by a discharge which is graded in frequency according to the degree of mechanical deformation. In general this discharge is slowly-adapting and can provide sensory information concerning maintained tactile stimulation. This is in contrast to hair receptors which signal principally rate of change of hair movement. Touch receptors of cat skin have discrete receptive spots which may bear similarity to the well known touch spots of human skin. Many touch units in the cat have multiple receptive spots, often located several millimetres apart. The morphology of such receptive spots in the skin of cat is not yet known. However, the discreteness of the touch receptive spots suggests that a given point on the skin could possibly be subserved by only one touch receptor fibre.

Of considerable interest is the fact that many touch units show discharge not only in response to mechanical stimulation but also to thermal stimuli. Although the responses to tactile stimuli are fairly consistent among touch units, the responses to thermal stimuli vary considerably among different units and even in a given unit may show considerable variability.

The majority of the touch units show considerable aperiodicity of discharge at a given skin temperature. Many show a more consistent behaviour to phasic changes than to static levels of skin temperature. As noted by Witt & Hensel (1959) the majority of the thermosensitive touch units have some of the properties of cold receptors as described in the lingual nerve (Hensel & Zotterman, 1951*a*). However, the highly reproducible responses to tactile stimuli, in contrast to the rather variable responses to thermal stimuli, suggest that touch units should be considered primarily as mechanoreceptors with a possible secondary function as thermoreceptors. Other examples of receptors responsive to both mechanical and thermal stimulation are known. Thus the pit receptors of *Crotalidae* are responsive to both infra-red radiation and to mechanical stimuli (Bullock & Diecke, 1956). Also, certain receptors of the tongue which are responsive to pressure also show discharge to rapid cooling (Hensel & Zotterman, 1951*b*).

The mechanism by which touch receptors are sensitive to both mechanical and thermal stimuli is not known. However, such dual sensitivity does occur and it seems clear that receptor specificity is less strict than has been generally assumed (cf. Sinclair, Weddell & Zander, 1952). Nevertheless some degree of specificity is indicated by the fact that hair receptors generally fail to respond to changes in skin temperature whereas most touch receptors do (Hunt & McIntyre, 1960).

It is possible that sensory information relating to both touch and temperature may be conveyed by the same fibres. At the present time the means whereby these two types of information might be decoded by the central nervous system can only be surmised. One factor that could be of importance is the fact that the frequencies of discharge associated with rather slow changes in skin temperature are generally considerably lower than those resulting from tactile stimulation. It is conceivable that collaterals of touch unit fibres could establish synaptic connexions with two types of second-order neurones. In one type of relay characteristics of synaptic transmission could limit response of the second-order neurone to the lower range of frequencies that are generally involved in responses of touch units to thermal stimuli. The other type of relay could transmit the higher frequencies generally associated with responses to mechanical stimuli and would be responsive predominantly to phasic changes in discharge. Such a scheme would not provide for complete differentiation of the two types of information.

At the present time the possibility that some small myelinated fibres convey temperature information exclusively cannot be excluded (see Maruhashi *et al.* 1952; Hunt & McIntyre, 1960). Also, the role of non-myelinated fibres in signalling temperature (Douglas, Ritchie & Straub, 1959; Iggo, 1959) must be considered in interpreting cutaneous temperature sensibility.

SUMMARY

By recording from single afferent fibres from the sural nerve of cat, the following characteristics of cutaneous touch receptors have been found:

1. Touch units display 1–5 discrete receptive spots and show a slowly-adapting discharge to mechanical displacement of such spots. Touch receptive spots may become blocked by repeated mechanical stimulation.

2. Many touch units show a base-line discharge, in the absence of mechanical stimulation, which is dependent in average frequency on skin temperature.

3. The responses of touch units to mechanical stimuli can be influenced by sufficient warming or cooling of the skin. In these circumstances the receptor becomes rapidly-adapting and fewer discharges are evoked by a constant mechanical stimulus.

4. The relation of these findings to the problem of specificity of receptors to thermal and mechanical stimuli is considered.

This work was supported by a research grant (B1320) from the National Institutes of Health.

REFERENCES

BULLOCK, T. H. & DIECKE, F. P. J. (1956). Properties of an infra-red receptor. *J. Physiol.* **134**, 47–87.

DOUGLAS, W. W., RITCHIE, J. M. & STRAUB, R. W. (1959). Discharges in non-myelinated (C) fibres in the cat's saphenous nerve in response to changing the temperature of the skin. *J. Physiol.* **146**, 47–48*P*.

HENSEL, H. & ZOTTERMAN, Y. (1951*a*). Quantitative Beziehungen zwischen der Entladung einzelner Kältefasern und der Temperatur. *Acta physiol. scand.* **23**, 291–319.

HENSEL, H. & ZOTTERMAN, Y. (1951*b*). The response of mechanoreceptors to thermal stimulation. *J. Physiol.* **115**, 16–24.

HUNT, C. C. & MCINTYRE, A. K. (1960). An analysis of fibre diameter and receptor characteristics of myelinated cutaneous afferent fibres in cat. *J. Physiol.* **153**, 99–112.

IGGO, A. (1959). Cutaneous heat and cold receptors with slowly conducting (C) afferent fibres. *Quart. J. exp. Physiol.* **44**, 362–370.

MARUHASHI, J., MIZUGUCHI, K. & TASAKI, I. (1952). Action currents in single afferent nerve fibres of the cat. *J. Physiol.* **117**, 129–151.

SINCLAIR, D. C., WEDDELL, G. & ZANDER, E. (1952). The relationship of cutaneous sensibility to neurohistology in the human pinna. *J. Anat., Lond.*, **86**, 402–411.

WITT, I. & HENSEL, H. (1959). Afferente Impulse aus der Extremitätenhaut der Katze bei thermischer und mechanischer Reizung. *Pflüg. Arch. ges. Physiol.* **268**, 582–596.

J. Physiol. (1960), **153**, *pp.* 99–112
With 8 text-figures
Printed in Great Britain

AN ANALYSIS OF FIBRE DIAMETER AND RECEPTOR CHARACTERISTICS OF MYELINATED CUTANEOUS AFFERENT FIBRES IN CAT

By C. C. HUNT and A. K. McINTYRE
From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.

(*Received* 14 *March* 1960)

The preceding paper considered the characteristics of touch receptors in the skin of cat, the analysis being made on certain of the fibres that were isolated in the course of the study now to be described. The present report is concerned with the relation between fibre diameter and receptor characteristics, as determined in 421 individual myelinated afferent fibres from the sural nerve of cat.

Several considerations caused us to undertake this problem. Although there have been many studies on perception of cutaneous sensation in man, comparatively few investigations have been concerned with the receptor characteristics of individual myelinated cutaneous afferent fibres in mammals (see Discussion). Another factor was that recent anatomical studies (Sinclair, Weddell & Zander, 1952) have suggested re-evaluation of the generally held 'doctrine of specific nerve energies', i.e. the specificity of a given receptor for a particular type of stimulus. Although it has long been recognized that the specificity of receptors for particular types of stimulus was not absolute, the concept that sensory information for a particular type of natural stimulus is transmitted exclusively in certain afferent fibres has been widely accepted.

The use of volleys in cutaneous nerves, graded in size by stimulus strength, for the study of spinal reflexes and of sensory systems has also posed a need for more information on the relation between receptor characteristics and fibre diameters. Similarly, the detailed information now available on responses to natural stimuli of cortical neurones of the primary sensory receiving area (Mountcastle, 1957) requires, for its fuller interpretation, more information as to receptive fields and response characteristics as recorded in primary afferent fibres.

It will be shown that several distinct categories of receptors may be recognized by their responses to adequate or natural stimulation, and the relation of fibre diameter to these categories has been defined. Also,

receptor characteristics have been more fully described and receptive fields of various types of receptors have been mapped.

METHODS

Adult cats anaesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories) were used. Laminectomy was performed, the appropriate dorsal roots (and associated ventral roots) were cut near the spinal cord, and nerves in the hind limb other than the sural were also cut. In most preparations single afferent fibres were isolated in dorsal root filaments. The presence of a fibre from the sural nerve was detected by an impulse evoked by stimulation of the latter. Conduction times were determined from stimulus–response intervals. The latter were measured by a Berkeley 1 MC counter (see Hunt & McIntyre, 1960*a*). The beginning of the counted interval was signalled by the stimulus, the end by the onset of the impulse. In all cases the stimulus–impulse interval was observed on one beam of a dual-beam Tektronix oscilloscope (model 502) and the gated interval simultaneously on the other beam. On completion of each experiment the conducting pathway between stimulating cathode and proximal recording electrode was excised and measured. From the conduction time, 0·1 msec was deducted for utilization time, and conduction velocity then calculated (Hunt, 1954). In measuring conduction time stimulus strength was made approximately twice threshold strength.

Exposed tissues were covered with paraffin oil initially equilibrated with 5% CO_2 and 95% O_2. Body temperature was kept between 37 and 39° C. The region of the skin innervated by the sural nerve was carefully clipped so that the hairs protruded about 1 mm from the skin. The limb was held by a pin inserted into the medial malleolus, so that the sural area was suspended in air.

RESULTS

Of the total of 421 single myelinated afferent fibres studied from sural nerve, the great majority exhibited discharge in response to some form of natural or adequate stimulation. Most afferent fibres and their receptors (units) studied fell into one of several distinct categories with respect to the type of stimulus most effective in eliciting discharge. The most commonly encountered fibres had receptors in which activity was evoked primarily by displacement of hairs, and have been designated 'hair units'. Another major category consisted of 'touch units', as described in the preceding paper (Hunt & McIntyre, 1960*b*). Fibres showing discharge in response to other types of stimulation included those responding to heavy pressure, to brief tap on the skin, to pinch or pin prick, as well as others in which discharge was evoked by stimulation of subcutaneous or joint structures. The response characteristics of the various types of units will be considered separately.

Since myelinated cutaneous fibres have a wide and characteristic distribution of fibre diameters, it was of interest to compare the distribution of calculated fibre diameters with the histological spectrum in order to assess the adequacy of sampling. For conversion of conduction velocity to diameter, direct proportionality by a factor of 6 has been used (Hursh, 1939). The calculated diameter distribution of the 421 fibres sampled is

shown in Fig. 1. According to the conversion used, the largest fibres would be in the 16–17 μ band, the peak of the α group at 10–12 μ, and the peak of the δ group at 3–4 μ. In general this distribution is similar to the histological spectrum shown in Fig. 8 of Gasser & Grundfest's (1939) paper. The peak of the δ group in the calculated fibre diameter spectrum is probably at too small a diameter; from histological spectra this would be expected to occur at slightly larger diameter. While it seems likely that conduction velocity (m/sec) is related to diameter (μ) by a factor of 6

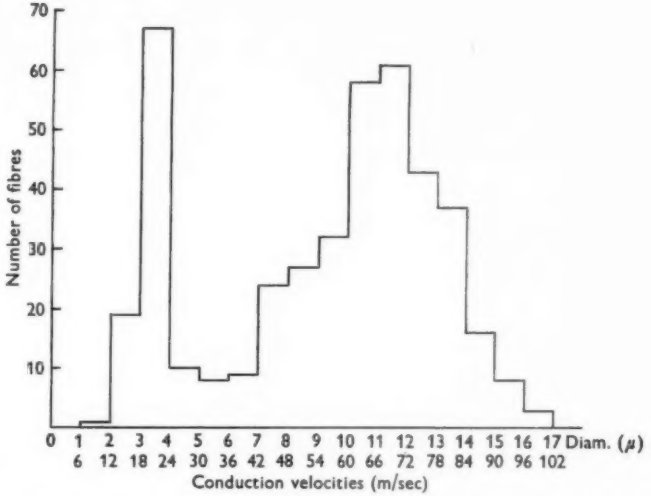


Fig. 1. Distribution of fibres according to conduction velocities and calculated diameters, total sample of 421 sural fibres.

in the largest fibres, the factor of conversion may deviate more widely among δ fibres. This could explain a difference in peak distribution of δ fibre diameters between the isolated fibre and histological spectra. In spite of this uncertainty, the calculated diameter distribution indicates that fibres throughout the myelinated range were sampled. Probably because of the greater ease of isolating larger fibres, the sample of α fibres may be disproportionately large. However, the sample may be considered representative of the spectrum of fibre diameters with the possible exception of the smallest δ fibres. The conduction velocities also suggest that the largest α fibres are considerably faster than Group II fibres of muscle nerves (Lloyd & Chang, 1948; Hunt, 1954). Also, the minimum in the distribution separating the α from the δ modes occurs at a larger diameter than that separating Groups II and III in muscle nerves. In the

present study fibres with calculated diameters between 6 and 17 μ will be called α, those between 1 and 6 μ δ (see Gasser, 1960).

Hair receptors

Units responding principally to hair movement numbered 263 of the total sample, = 62%. Movement of hairs was the most effective means of eliciting discharge. Hair movement was produced by brushing the hairs, which had been clipped short, with a fine hairbrush, with a cat's vibrissa, or by displacing hairs with fine watch-maker's forceps. Movement of hair without otherwise deforming the skin served to evoke discharge, in contrast to the touch units considered below. A typical response of a hair unit is shown in Fig. 2.

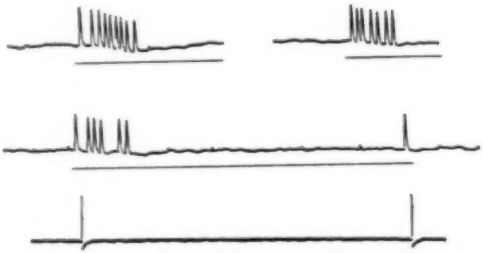


Fig. 2. Discharge in a hair receptor fibre to bending of hairs. Bar gives estimate of duration of bending. Time marker, 100 msec, lowest trace.

Hair units were always rapidly-adapting and ceased discharging when hairs were maintained in the bent position. However, some were so sensitive that slight movement of hairs while in the bent position would result in discharge. Although all were rapidly-adapting, differences in the rate of adaptation were evident among the various hair units. Thus the most rapidly-adapting responded only when the hair movement was rapid and not to slow bending of hairs. Others discharged even when hairs were moved slowly.

The calculated diameter distribution of fibres of hair units is shown in Fig. 3. The distribution is clearly bimodal, with peaks in the α and δ ranges. Comparison with Fig. 1 reveals that hair units accounted for the bulk of the δ group and a very substantial fraction of the α group. Also, the largest fibres in the α range were from hair receptors. Hair units appear to occur over the entire myelinated fibre range, from small δ to largest α, and there seems to be no band of fibre diameters free of fibres from hair receptors.

The receptive fields of hair units are shown in the charts of Fig. 4. A total of 152 units are shown on six projections of the region of skin

innervated by the sural nerve. This number of hair units was contained in a sample of about 300 sural fibres. Assuming the sural nerve to have about 1200 fibres (an unpublished count (Gasser, personal communication) of the myelinated fibres in sural nerve totalled 1073), one may

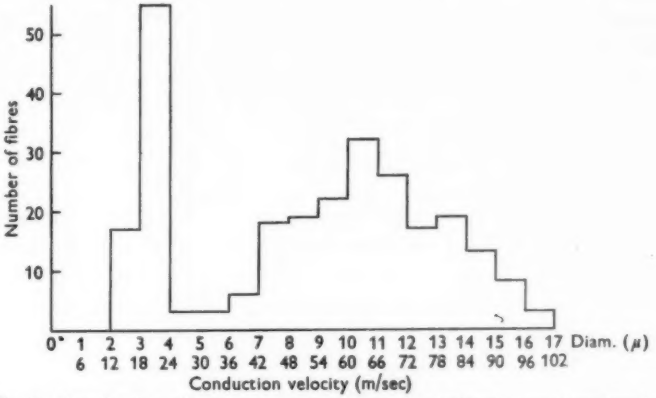


Fig. 3. Distribution of conduction velocities and calculated fibre diameters in hair units.

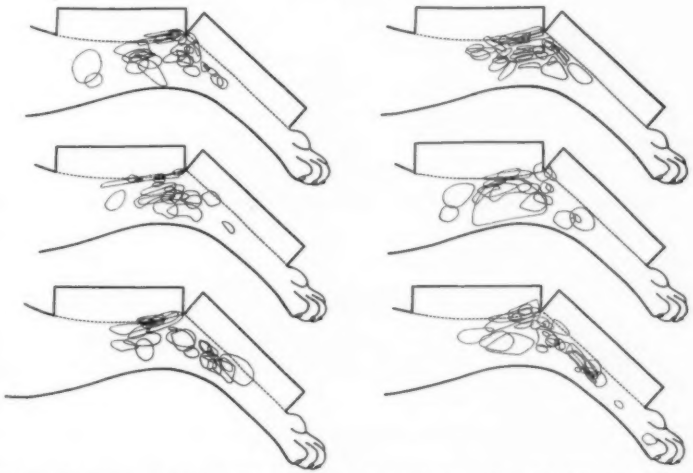


Fig. 4. Receptive fields of 152 hair units. Lateral view of cat hind limb and foot shown, with flaps indicating skin medial to mid line (indicated by dotted line).

obtain a rough estimate of the density and degree of overlap of hair-receptive fields in the sural region of a cat by combining the projections of Fig. 4 and multiplying by a factor of approximately 4. In this manner

it would be possible to estimate the approximate number of hair units excited by movement of hairs in a given area. Actually the density of innervation of hair units would be slightly greater than this estimate, since the data have been derived from a number of animals in which the region supplied by the sural nerve is not exactly the same.

The areas of receptive fields of individual hair units were estimated by drawing to scale on graph paper the outline of each unit's field. The individual fields were then cut out and weighed and their areas estimated by

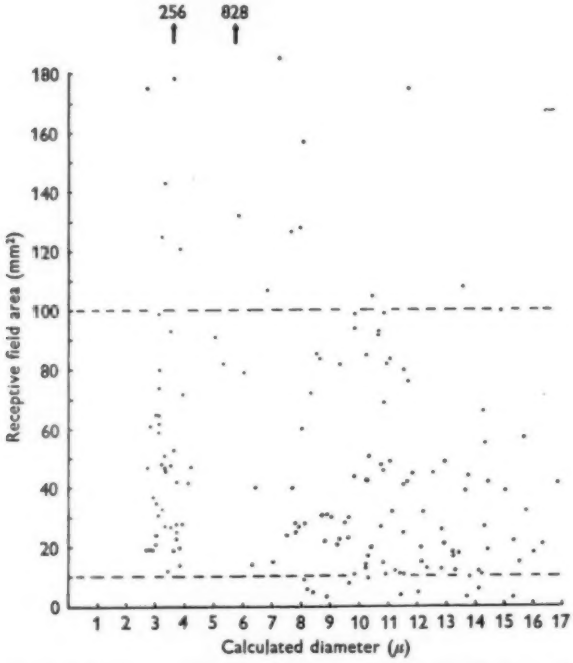


Fig. 5. Relation between receptive-field area and diameter of afferent fibre of the same hair units shown in Fig. 4. Interrupted horizontal lines at receptive-field areas of 10 and 100 mm².

comparison with the weights of standard areas. The receptive-field areas showed wide variations, the smallest being about 3 mm² and the largest 828 mm². The latter was quite exceptional, and all but two units had field areas between 3 and 185 mm². A plot of receptive-field areas of 156 hair units is shown as a function of calculated fibre diameter in Fig. 5. Although there is wide scatter, two features are clear: there was a greater incidence of large receptive fields among smaller fibres, and the smallest

receptive fields (less than 10 mm²) were found only in fibres of α diameter. However, except for these features there was little correlation between receptive-field area and fibre diameter.

The sensitivity of hair receptors varied considerably. Some were so sensitive that very slight movement of the skin transmitted from a considerable distance would evoke discharge. For example, a number of units were found, with receptive fields central to the lateral malleolus, which responded when the foot was moved a small distance. Such units showed discharge to gentle blowing on the skin. Great care was necessary to define the limits of distribution of the hair receptors of such units. At the other extreme were units which responded only when the hairs were briskly moved, for example, by rapid stroking with a brush. Between these extremes there were many units with intermediate sensitivities. In general, if a unit showed great sensitivity the entire receptive field exhibited this characteristic and, conversely, in a less sensitive unit movement of various hairs within the receptive field indicated the same order of sensitivity. However, some differences in sensitivity within the field of a given unit could sometimes be detected, particularly in units of intermediate sensitivity.

In a number of hair units an estimate was made of the receptor sensitivity, grading on a basis of from 1 to 4 plus. While such estimates are only approximate, the range of sensitivity was sufficiently broad to give it significance. A significant correlation was found between sensitivity and fibre diameter, indicating that the fibres of slower conduction velocity and smaller diameter tend to have the more sensitive receptors. In this regard it may be recalled that Zotterman (1939) noted that very light stroking of the skin elicited discharge confined to fibres considered to be in the δ range.

Spontaneous discharge was rarely found in hair units. A few were noted which gave an impulse associated with each heart beat. In some cases this occurred only when the responsive hairs were bent to a certain position. No background discharge of the kind found in touch units (see below) was noted in hair units. Also, hair receptors were usually unresponsive to temperature changes of the skin although on rare occasions hair units showed a few discharges when the skin temperature was raised above 41–42° C. In contrast to touch units (Hunt & McIntyre, 1960*b*), hair receptors did not develop non-responsiveness after repeated mechanical stimulation of the skin.

Touch receptors

Units showing discharge to light touch of the skin numbered 114 or 27% of the total sample. Their responses to both mechanical and thermal stimulation have been described in the previous paper (Hunt & McIntyre,

1960*b*). As was there noted, they could usually be readily differentiated from hair receptors and other mechanoreceptors.

The calculated diameter distribution of touch units is shown in Fig. 6. In contrast to that of hair units, touch receptor fibres have a unimodal distribution which is essentially confined to the α group. In the α range the distributions of hair and touch receptor fibres overlap considerably,

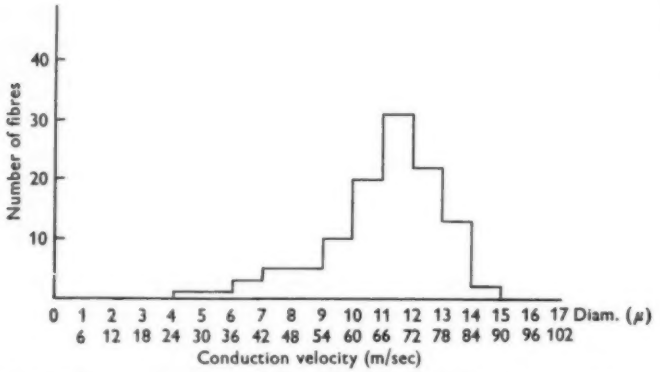


Fig. 6. Conduction velocity and calculated diameter distribution of 114 touch unit fibres.

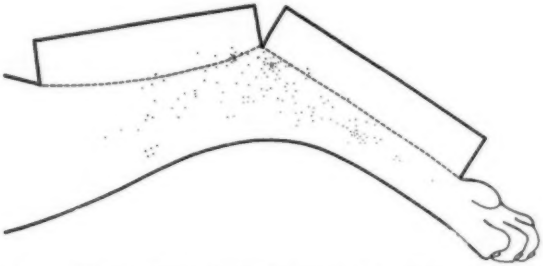


Fig. 7. Distribution of receptive spots of 77 touch units. Projection similar to those of Fig. 4.

although the largest touch units are appreciably smaller than the largest hair fibres.

The distribution of receptive spots of 77 touch units is shown in Fig. 7. Multiplication of the number of receptive spots shown by about 4 (see above) would indicate the approximate density of innervation by this type receptor. It is clear that the density of touch receptor spots is high. However, the spots are so discrete that a given point on the skin may well be served by only one receptor.

Pressure receptors

A total of 21 units were isolated which showed discharge on pressure to the skin of considerably greater intensity than that required to evoke impulses from the touch receptors noted above. However, the degree of pressure necessary to cause discharge in pressure units was judged less than that which would be noxious. The discharge was sustained when pressure was maintained. Hence these receptors may be considered slowly-adapting. While it was not possible to define precisely the limits of a receptive field, pressure was effective only when applied to a fairly limited area. In most cases the receptive area shifted with movement of the skin,

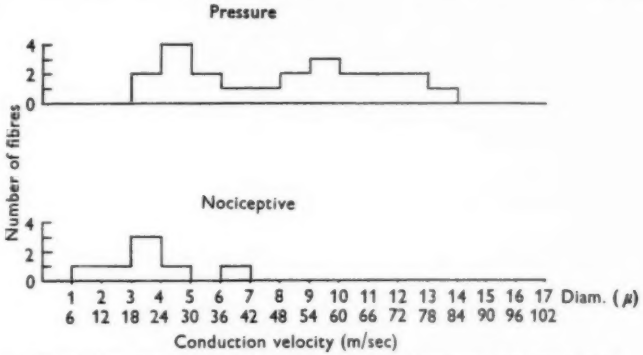


Fig. 8. Conduction velocity and calculated diameter distributions of pressure unit fibres and of fibres from presumed nociceptive units.

but in occasional units the receptors did not appear to follow skin movement and were judged to be subcutaneous. However, even units which appeared to shift their receptive areas with skin movement might have been located deep to the skin itself.

The diameters of pressure receptor fibres were scattered from 3 to 14μ with no distinct mode (Fig. 8). They thus occur in both the α and δ fibre groups. Unlike the touch receptors, pressure receptors did not show spontaneous or background discharge and did not respond to thermal stimuli.

Probable nociceptive receptors

A total of seven fibres were isolated which showed discharge only to heavy pinching of the skin or pin-prick, stimuli judged to be noxious in character. Those responding to pinching of the skin were often slowly-adapting, discharge being maintained for the duration of the stimulus. Units responding to pin-prick frequently gave only a few impulses on pricking the skin or scratching the skin surface with a pin. No after-

discharge was seen, in contrast to that found in nociceptive non-myelinated units (Zotterman, 1939).

The presumed nociceptive units were essentially confined to the δ range of fibre diameters (Fig. 8). The relatively small number of such units may be accounted for, in part, by the fact that severe handling of the skin was minimized because it led to block of touch receptors. However, even if the six δ units which were unidentified as to receptor function were considered as possibly 'nociceptive', the total number of nociceptive units would be small relative to the number of δ hair units.

Other receptors

A few units with receptor characteristics different from the above were encountered. These included five units which responded only to a brisk tap on the skin, usually with only one or two impulses. These units had fibres between 8 and 14μ and may possibly have been nearly blocked touch receptors. Another category consisted of two units responding to mechanical stimulation of the ankle joint or associated ligamentous structures. One of these was in the δ range (4·1μ), the other in the α band (10·5μ). One unit (calculated diameter 11·7) had its receptor in the fascia of the gastrocnemius muscle. It gave a sustained discharge to pulling on the fascia.

Of the total sample of fibres eight were isolated which showed no response to mechanical stimulation; the three of these that were examined also failed to respond to warming or cooling of the skin. As was noted above, six of the unidentified units were in the δ range and could possibly be nociceptive. The remaining two unidentified units were of the α band and might have been blocked touch receptors.

DISCUSSION

The present study indicates that the most common receptor type among myelinated fibres in the sural nerve of cat is activated primarily by movement of hairs. Responses to this type of stimulation have been noted previously by Adrian (1930) and by Zotterman (1939), both of whom described impulses of both large and small size when recording from a cutaneous nerve and lightly stroking the skin. Zotterman (1939) utilized relative spike potential amplitude as an index of fibre diameter and concluded that receptors of smaller fibres (judged to be δ) were activated by lighter touch than that required to initiate discharge in larger fibres. While he did not relate such stimulation specifically to hair receptors, his findings are supported by the present results, which indicate that the lowest threshold hair receptors tend to have fibres of small diameter. Maruhashi,

Mizaguchi & Tasaki (1952) also reported responses in fibres of saphenous nerve of cat related to hair movement. The relative number of hair receptors found was considerably less than in the present study, and the range of diameters was from 6 to 12 μ. The smaller fibres which they designated as 'wide receptive field', ranging in size from 2 to 5 μ, are probably the same as those here reported as δ hair units.

Receptors responding to touch of one or two spots on the skin were noted by Maruhashi *et al.* (1952) and found to be associated with fibres between 8 and 14 μ in diameter. The previous paper (Hunt & McIntyre, 1960*b*) provided a more detailed description of their behaviour and indicated that these receptors are generally slowly-adapting. The finding that the same fibres may show discharge to mechanical stimulation of the skin and changes in frequency on variation of skin temperature has recently been reported by Witt & Hensel (1959). While the latter authors stated that some such units showed localized areas of mechanical sensitivity, the mechanoreceptive characteristics of these fibres were not described in detail. The experiments reported here show that touch units alone, among the mechanoreceptors of skin innervated by myelinated fibres, exhibit modulation of background discharge by variations in skin temperature.

Two aspects of the distribution of receptor types in the present sample of myelinated fibres are surprising. One is the relative paucity of units responding to nociceptive stimulation. As noted in Results (p. 107), the unidentified units in the δ range could possibly have been nociceptive. Even including these the relative number would be small. Another potential deficit in sampling may be that relatively few of the smallest δ fibres might have been isolated and these could have been nociceptive in function. One problem in assessing the adequacy of the sample in the 1–3 μ range is the uncertainty of the factor converting conduction velocity to diameter in this range. There are still other difficulties in evaluating what is a nociceptive stimulus. The mechanical stimuli employed may be so intense as to be capable of initiating impulses in nerve fibres themselves. While the present sample shows nociceptive units confined to the δ diameter range, it might be possible to stimulate directly the fibres of blocked touch units and so erroneously to conclude they were nociceptive. In spite of the reservations expressed above, it seems clear that the bulk of the δ fibres are from hair receptors and that nociceptive units must constitute only a small fraction of the δ group of fibres.

The reservation expressed as to adequacy of sampling of the smallest δ fibres may also be applicable to the apparent lack of fibres concerned solely with thermoreception. Maruhashi *et al.* (1952) reported the isolation of a number of fibres in cat cutaneous nerve with directly measured diameters of 1·5–3 μ which showed discharge to cooling the skin but not to mechanical

stimulation. Such fibres might not have been detected in the present study. From the present results the possibility cannot be excluded that myelinated fibres other than those of touch units may convey information concerning skin temperature. In addition to the myelinated fibres, non-myelinated fibres have thermosensitive receptors (Iggo, 1958, 1959; Douglas, Ritchie & Straub, 1959).

The diameter distributions of cutaneous myelinated afferent fibres of different receptor types are of importance in evaluating the central effects of graded afferent volleys in cutaneous nerves. Hair receptor fibres are distributed throughout the α and δ fibre diameter ranges. Hence volleys of increasing size, from those including only a few of the largest to those in which the entire population of myelinated fibres is activated, will involve increasing numbers of hair units. In contrast, once a volley occupies all of the α fibres a further increase in stimulus strength to include δ fibres would be expected to recruit activity in very few additional fibres from touch receptors. The considerable overlap in diameters of fibres from receptors of various types would make it very difficult to relate reflex actions evoked by volleys of graded size to specific receptors.

One factor of importance in interpreting the effects of natural stimulation of the skin is the large overlap of receptive fields of hair units. Movement of hairs in a restricted area of the cat's skin is certain to activate hair receptors of a number of hair units. In contrast, the touch units may show little overlap because of the discreteness of their receptive spots. With regard to the touch receptor, information as to tactile stimulation must often be derived by the central nervous system from an increment in discharge superimposed upon a temperature-dependent background discharge. This background discharge must also be considered in experiments in which cutaneous nerves are left intact, for in such circumstances there is an appreciable input into the spinal cord from this source.

While doubt has been expressed as to the specificity of receptors based on the lack of morphological differentiation seen in nerve endings in certain areas of skin (Sinclair *et al.* 1952), it seems clear from the present results as well as those of previous studies (Zotterman, 1939; Maruhashi *et al.* 1952) that some degree of receptor specialization exists. Thus, cutaneous afferent fibres can be isolated which show responses only to particular kinds of mechanical stimulation. The fact that touch receptor units display a discharge which is dependent on skin temperature as well as showing responses to tactile stimulation (Witt & Hensel, 1959; Hunt & McIntyre, 1960*b*) suggests that the specificity is not as absolute as was generally considered. However, the lack of such temperature responses in hair units indicates a degree of functional differentiation between hair and touch receptors. A reasonable view in the light of available evidence would

appear to be that some degree of receptor specificity exists but that possibly in some fibres information relating to more than one type of cutaneous sensation may be transmitted.

Evidence as to cutaneous receptors in cat skin must be interpreted with caution in relation to the problem of sensory modalities in man. It is quite possible that the relative proportions of various receptor types may differ considerably between cat and man. In particular, the greater amount of hair in an animal such as cat might be associated with a greater density of hair receptors. The large amount of overlap in receptive fields of hair units in the cat suggests that localization of a stimulus which activates only this type of receptor would require a central mechanism that could decipher this information from relative frequencies of discharge in a number of fibres. On the other hand, from the standpoint of peripheral organization alone, touch units could signal spatial discrimination in a simpler fashion. In view of the recent evidence that receptors of non-myelinated fibres also signal touch (Douglas & Ritchie, 1957; Iggo, 1958; see also Zotterman, 1939), activity from such receptors must also be considered in evaluating peripheral mechanisms of cutaneous tactile sensibility.

SUMMARY

A study of conduction velocity and discharge pattern of 421 isolated myelinated afferent fibres in the sural nerve of cat indicates the following:

1. Hair receptor fibres were the most numerous, accounting for the bulk of the δ fibres and a large proportion of α fibres. They accounted for the largest fibres in the sural nerve.

2. Receptive fields of hair units have been determined and show a large amount of overlap. Receptive field areas varied widely and there was some tendency for the smaller fibres to have larger receptive fields.

3. Hair receptors were invariably rapidly-adapting and varied in sensitivity to natural stimulation, the smaller fibres tending to have the more sensitive receptors.

4. Touch receptor fibres (Hunt & McIntyre, 1960*b*) were essentially confined to the α diameter band, the largest being less in diameter than the hair receptor fibres of greatest diameter.

5. Units responding to pressure greater than that required to activate touch receptors were distributed widely in diameter. Such units showed slow adaptation. Some appeared to be located in the skin, others subcutaneously.

6. A variety of other receptor types were noted. These included seven which were presumably nociceptive, five responding to brief tap of the skin, two joint units, and eight in which discharge could not be elicited by the stimuli employed.

Preliminary experiments were carried out at the Albert Einstein College of Medicine, New York; the assistance of Dr Calvin Ackerman in these is gratefully acknowledged. The work was supported by a research grant (B 1320) from the National Institutes of Health.

REFERENCES

ADRIAN, E. D. (1930). Effects of injury on mammalian nerve fibres. *Proc. Roy. Soc.* B, **106**, 596–618.

DOUGLAS, W. W. & RITCHIE, J. M. (1957). Non-medullated fibres in the saphenous nerve which signal touch. *J. Physiol.* **139**, 385–399.

DOUGLAS, W. W., RITCHIE, J. M. & STRAUB, R. W. (1959). Discharges in non-myelinated (C) fibres in the cat's saphenous nerve in response to changing the temperature of the skin. *J. Physiol.* **146**, 47–48*P*.

GASSER, H. S. (1960). Effect of the method of leading on the recording of the nerve fiber spectrum. *J. gen. Physiol.* **43**, 927–940.

GASSER, H. S. & GRUNDFEST, H. (1939). Axon diameters in relation to the spike dimensions and the conduction velocity in mammalian A fibers. *Amer. J. Physiol.* **127**, 393–414.

HUNT, C. C. (1954). Relation of function to diameter in afferent fibers of muscle nerves. *J. gen. Physiol.* **38**, 117–131.

HUNT, C. C. & MCINTYRE, A. K. (1960*a*). Characteristics of receptors from flexor longus digitorum and the adjoining interosseous region of cat. *J. Physiol.* **153**, 74–87.

HUNT, C. C. & MCINTYRE, A. K. (1960*b*). Properties of cutaneous touch receptors in cat. *J. Physiol.* **153**, 88–98.

HURSH, J. B. (1939). Conduction velocity and diameter of nerve fibers. *Amer. J. Physiol.* **127**, 131–139.

IGGO, A. (1958). Single C fibres from cutaneous receptors. *J. Physiol.* **143**, 47–48*P*.

IGGO, A. (1959). Cutaneous heat and cold receptors with slowly conducting (C) afferent fibres. *Quart. J. exp. Physiol.* **44**, 362–370.

LLOYD, D. P. C. & CHANG, H. T. (1948). Afferent fibers in muscle nerves. *J. Neurophysiol.* **11**, 199–208.

MARUHASHI, J., MIZAGUCHI, K. & TASAKI, I. (1952). Action currents in single afferent nerve fibres of the cat. *J. Physiol.* **117**, 129–151.

MOUNTCASTLE, V. B. (1957). Modality and topographic properties of single neurons of cat's somatic sensory cortex. *J. Neurophysiol.* **20**, 408–434.

SINCLAIR, D. C., WEDDELL, G. & ZANDER, E. (1952). The relationship of cutaneous sensibility to neurohistology in the human pinna. *J. Anat., Lond.*, **86**, 402–11.

WITT, I. & HENSEL, H. (1959). Afferente Impulse aus der Extremitätenhaut der Katze bei thermischer und mechanischer Reizung. *Pflüg. Arch. ges. Physiol.* **268**, 582–596.

ZOTTERMAN, Y. (1939). Touch, pain and tickling, an electrophysiological investigation on cutaneous sensory nerves. *J. Physiol.* **95**, 1–28.

J. Physiol. (1960), **153**, *pp.* 113–126
With 9 text-figures
Printed in Great Britain

A QUANTITATIVE STUDY OF SENSITIVE CUTANEOUS THERMORECEPTORS WITH C AFFERENT FIBRES

BY H. HENSEL, A. IGGO* AND INGRID WITT
From the Department of Physiology, Marburg/Lahn, Germany

(*Received* 16 *March* 1960)

It has been demonstrated that heating or cooling the cat's skin can elicit a discharge of impulses in slowly-conducting (C) afferent fibres (Iggo, 1958*b*, 1959*a*, *b*; Douglas, Ritchie & Straub, 1959; Douglas & Ritchie, 1959). However, it was not clear whether the C group contained fibres from 'specific' thermoreceptors comparable in sensitivity with those in the cat's tongue (Hensel & Zotterman, 1951*a*; Dodt & Zotterman, 1952). Most of the C fibre thermoreceptors hitherto found responded only to rather extreme heating or cooling (Iggo, 1959*a*, *b*) and therefore were possibly concerned with pain. They were relatively insensitive to mechanical stimulation. There are also numerous C fibres which respond both to mild mechanical stimulation and to sudden cooling of the skin (Douglas *et al.* 1959; Iggo, 1959*a*). They have been classified as mechanoreceptors by Iggo (1960).

In the present investigation sensitive specific cold and warm receptors with afferent C fibres have been found and their behaviour has been studied quantitatively. The opportunity has also been taken to examine quantitatively the responses of C heat receptors (Iggo, 1959*b*) and of a few C mechanoreceptors to thermal stimulation. A preliminary account of these results has been published (Hensel, Iggo & Witt, 1959).

METHODS

The cats used were anaesthetized with chloralose (60 mg/kg intramuscular) and urethane (250 mg/kg intramuscular). After depilation the leg was fixed by drill pins inserted through each end of the tibia. The skin over the saphenous nerve was incised, the nerve was ligated proximally and dissected free from connective tissue. The cut edges of the skin were tied to a metal ring and the trough formed was filled with liquid paraffin B.P. Fine strands of the nerve were dissected on a black Perspex plate and laid on silver electrodes. A pair of stimulating electrodes was placed under the nerve about 3 cm distal to the recording electrodes and an earthed plate was placed between the two pairs of electrodes. The amplified impulses in the fine nerve strand were recorded by means of two separate systems, each consisting of a cathode-ray oscilloscope and a recording camera. The first system recorded the impulses in the usual way with a stationary cathode-ray beam and moving bromide paper (Fig. 1*A*). The expanded sweep of the second oscilloscope was triggered by the square-wave pulse

* On leave from Physiology Department, University of Edinburgh.

generator used for electrical stimulation of the nerve, and allowed an exact measurement of conduction velocity. Photographic records of the moving beam were taken on slowly moving bromide paper (Fig. 1*B*). In order to test mechanical sensitivity, the skin was stroked or pressed with a wooden pin or touched with cotton wool. Calibrated hairs of various stiffness were used for more quantitative stimulation.

Quantitative thermal stimulation was accomplished by a water-circulated thermode of 2 cm diam., which was placed on the receptive field of the afferent fibre. The water was supplied by 4 ultra-thermostats set at various constant temperatures (±0·02° C). By means of a special switch each thermostat could be connected with the thermode, and constant temperatures or rapid thermal changes could thus be applied to the skin. The thermode allowed thermal changes to be imposed without any alteration in the mechanical stimulus. A fine thermocouple was placed on the receptive field and the temperature was recorded synchronously with the deflexions of the first oscilloscope with a special recording camera (Hensel, 1953). In some experiments thermal radiation was also used for heating the skin.

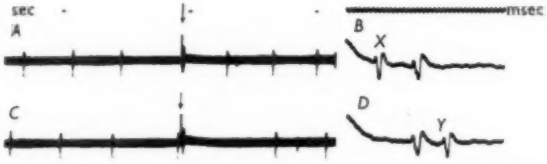


Fig. 1. The identification of the active cold fibre in a multi-fibre strand of the saphenous nerve.

A, Spontaneous discharge of a single C cold fibre and effect of electrical stimulation of the saphenous nerve (fibre No. 1). Arrows mark stimulus artifacts in *A* and *C*. *B*, Record with expanded sweep corresponding to that part of *A* marked by arrow. The first impulse (marked *X*) is the spontaneous cold fibre discharge, the second is from another C fibre excited by electrical stimulation. *C*, The same discharge as in *A*, but with stronger electrical stimulation. There is now a gap in the series of impulses. *D*, Expanded sweep. Both impulses were caused by electrical stimulation, the first is identical with the second in the upper record, the second is in the cold fibre. The conduction velocity was 1·1 m/sec. (See text for further explanation.)

RESULTS

Identification of single C fibres

This was based on the 'collision' technique (Iggo, 1958*a*), which makes it possible to measure conduction velocity in the one active fibre of a strand containing other fibres capable of conducting but not active. Figure 1 shows a record, taken with two oscilloscopes, of the impulses in a thin strand of the saphenous nerve. The steady discharge of a single cold fibre (fibre No. 1) in Fig. 1*A* and *C* was caused by a constant skin temperature of 25° C. During the natural discharge two electric shocks were sent into the saphenous nerve. The upper pair of tracings (Fig. 1*A* and *B*) shows a failure of the electrically produced impulse to interfere with the train of impulses from the receptor; the expanded sweep, Fig. 1*B*, shows one of the ascending impulses first (marked *X* in the figure) followed by an impulse in a different fibre caused by the shock to the nerve.

The lower pair of tracings shows in Fig. 1*C* a gap in the train of impulses. This appears immediately after the shock artifact and was caused by collision of the electrically produced antidromic impulse, corresponding to *Y*, with the natural impulse from the receptor. The expanded sweep, Fig. 1*D*, shows two impulses; the first is identical with the second impulse in the upper record and the second, *Y*, is an impulse in the cold fibre caused by the shock to the saphenous nerve. The similarity of this impulse to the first impulse in the upper tracing is further evidence that the second impulse in the lower tracing is in the cold fibre. The time between electrical stimulus and recorded impulse was 28 msec and the conduction distance was 31 mm, so that the conduction velocity turned out to be 1·1 m/sec.

In some experiments the slowly conducting fibres were identified only by the shape and duration of the C impulse and the typical sound in the loudspeaker.

Receptors excited by cooling the skin

Several C fibres responded to moderate cooling of the skin with an increase in frequency, and to heating with a decrease or complete inhibition, of the discharge. Within a certain range of constant temperatures a steady discharge was observed. These cold fibres were not excited by the mechanical stimuli used. In some examples the size of the receptive field was assessed by stimulation with small thermodes. The receptive fields were not larger than a few square millimetres.

The impulses in a single C fibre when the skin was cooled are shown in Fig. 2. A steady discharge can be seen at the constant initial temperatures (Fig. 2*C*, *D*, *E*). During cooling the impulse frequency rose rapidly, reached a maximum within the first second and then declined gradually. In the records *B*–*E* a slight transient temperature rise of about 1° C just before cooling led to a complete inhibition of the steady discharge.

The discharge frequency of another single C fibre (fibre No. 3) on cooling and rewarming the skin is shown in Fig. 3. The initial temperatures between 31 and 33·5° C corresponded to an indifferent temperature sensation in man. Even very slight cooling from 33 to 32·3° C increased the frequency from 1 to 22 impulses/sec, whereas warming by about a tenth of a degree caused the discharge to stop immediately. The 'dynamic' sensitivity of this cold receptor was 30 impulses/sec. ° C. This means that sudden cooling of 1° C would raise the impulse frequency by 30 impulses/sec. At indifferent temperatures a discharge was elicited by cooling of only 0·2° C. This was the final value of the external temperature change; the actual cooling when the first impulse was recorded was smaller than 0·1° C.

At constant temperatures the cold receptors exhibited a steady discharge, the frequency being a function of absolute temperature. The steady

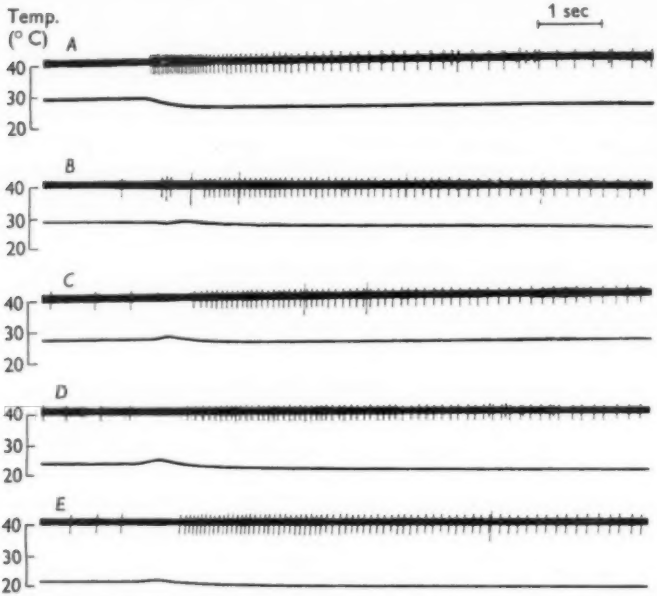


Fig. 2. Afferent impulses of a single C fibre and skin temperature when cooling the skin (fibre No. 1). Conduction velocity 1·1 m/sec. *A*, Cooling from 29 to 25·5° C; *B*, 29·3–28° C; *C*, 28·0–26·5° C; *D*, 24·5–22·5° C; *E*, 22–20·5° C.

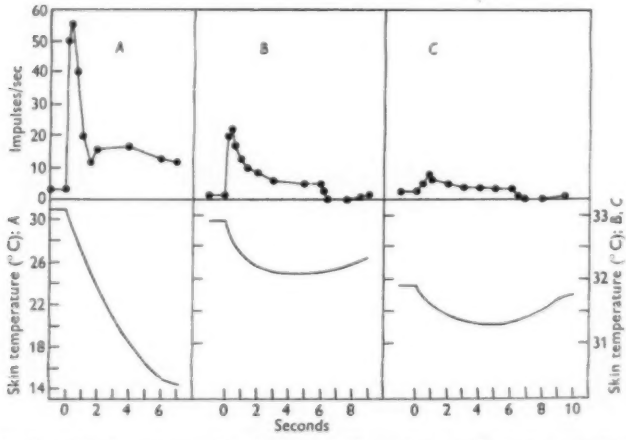


Fig. 3. Impulse frequency of a single cold fibre when cooling and warming the skin (fibre No. 3). The conduction velocity of the fibre was 1·5 m/sec. The left-hand temperature scale refers to *A*, and the right-hand scale to *B* and *C*.

discharge of the cold fibre No. 3 at constant temperatures between 28·8 and 14·2° C is shown in Fig. 4, and the steady discharge frequency is plotted against temperature in Fig. 5. Curve 1 was obtained by lowering the constant temperature stepwise from 37 to 14° C, keeping the temperature constant for 3 min after each step. Curve 2 was the result of the reverse procedure, starting at the lowest temperature. As the two curves are not identical it is probable that the adaptation time of 3 min was not

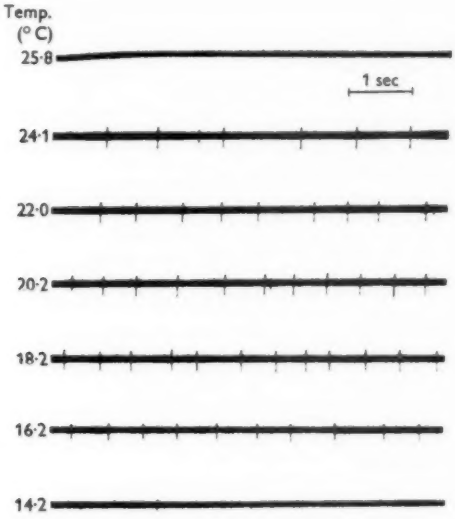


Fig. 4. Steady discharge of impulses in the single *C* fibre shown in Fig. 3 (fibre No. 3) at various constant temperatures of the skin.

quite sufficient to reach the final value of the steady discharge. The maximum frequency was reached at temperatures of 16·5 and 19·5° C, respectively. The width of the maximum varies considerably for different fibres (Fig. 6). Whereas fibre No. 3 has a well defined maximum, the cold fibre No. 1 exhibits practically the same impulse frequency over a temperature range of more than 10° C.

Receptors excited by heating the skin

C fibres have been found which could be excited by an external warming of only 0·3° C. This was the final value of temperature rise, the actual value at the first impulse being less than 0·1° C (Fig. 7*A*). Cooling by the same amount (Fig. 7*B*) inhibited the discharge completely. Most of these warm fibres were not excited by the mechanical stimuli used; one fibre had a

mechanical threshold of 2 g when touching the receptive field with a von Frey hair. There was no reaction of the warm fibres on sudden cooling from 30–33° C to low temperatures (10° C).

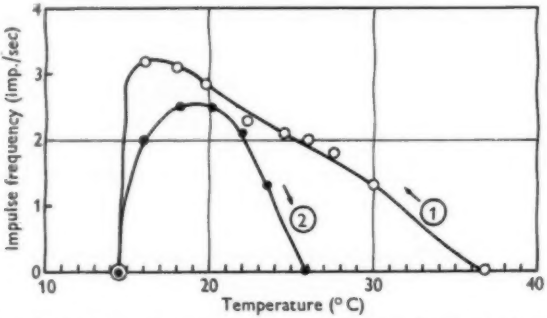


Fig. 5. Steady discharge frequency of a single C fibre (fibre No. 3), excited by cooling, at various constant temperatures. 1, Starting from high temperatures; 2, starting from low temperatures.

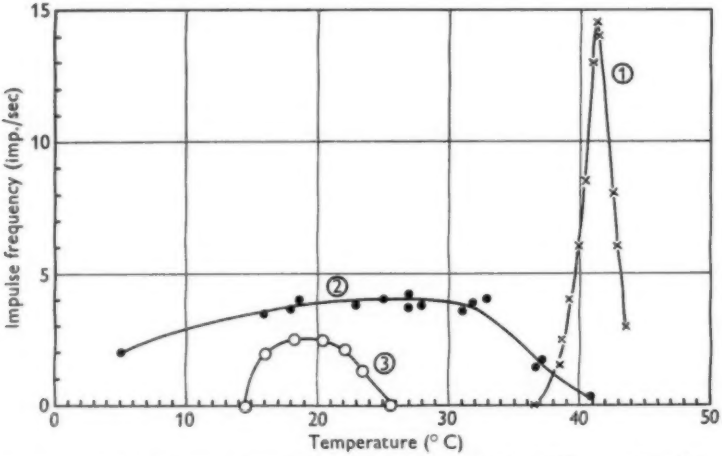


Fig. 6. Steady discharge frequency of three different single C fibres as a function of constant skin temperature. 1, Fibre excited by warming, fibre No. 8; 2, fibre excited by cooling, fibre No. 1; 3, fibre excited by cooling, fibre No. 3.

The frequency of discharge in a single warm fibre during various temperature changes, starting from indifferent initial temperatures between 31 and 33·5° C, is shown in Fig. 8. At a constant temperature of 40° C (last curve) a steady discharge is seen. Slight cooling of a few tenths of a degree stopped the discharge; slight warming by the same amount made the

discharge reappear. The dynamic sensitivity of this warm receptor was +33 impulses/sec.° C, which is of the same order as that of the C cold receptor. The maximum frequency of discharge and the time course of adaptation was also similar to that of the cold fibres (cf. Fig. 3).

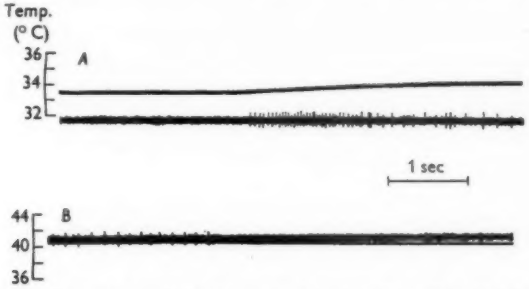


Fig. 7. Afferent impulses recorded in a single warm fibre (fibre No. 7) and the skin temperature, during slight warming and cooling of the skin.

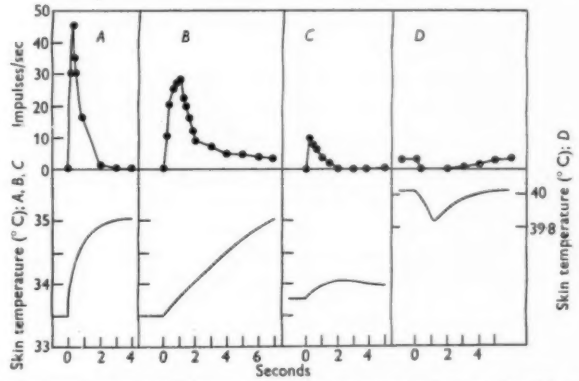


Fig. 8. Frequency of the discharge of impulses in a single C fibre (fibre No. 7), from a warm receptor, during cutaneous temperature changes. The left-hand temperature scale applies to *A*, *B* and *C* and the right-hand scale to *D*.

The warm fibre with the highest sensitivity exhibited a steady discharge at constant temperatures between 38·5 and 43° C. It fired a regular sequence of impulses, and showed a very sharp maximum at 41·2° C (Fig. 6, curve 1).

A number of non-myelinated fibres were found which could only be excited by heating the skin from indifferent temperatures (30–33° C) to more than 37° C. These probably correspond to the C heat receptors described by Iggo (1959*b*). In some cases, only a slow phasic discharge occurred during the temperature rise; other fibres also fired at high constant

temperatures, some with periodical bursts of impulses. One fibre with a high maximum frequency of 80 impulses/sec was silent at temperatures above 58° C; the inhibition was reversible. No other fibres were tested at these high cutaneous temperatures.

In order to prevent damage of the skin by the high temperatures, the thermal stimuli were maintained for not longer than about 30 sec. Under these conditions the threshold temperatures for a steady discharge of at least 30 sec duration varied from 38·5 to above 48° C in various fibres. Since the constant temperatures were maintained only for a short period of time, the values obtained may possibly not represent the true thresholds for the steady discharge.

Two of these less sensitive warm fibres were excited by the mechanical stimuli used. One fibre reacted to pressing the finger against the skin with a maximum discharge of 35 impulses/sec; on heating, the same fibre reached a maximum frequency of 44 impulses/sec.

Some features of 18 non-myelinated cold and heat fibres are summarized in Table 1. The temperature ranges for the steady discharge of the warm fibres 8 to 18 may not be correct, because the adaptation time was only 30 sec.

Receptors excited by mechanical stimulation

Cutaneous mechanoreceptors with afferent C fibres are more easily found than thermoreceptors, since the number of mechanosensitive nerve endings seems to exceed that of thermoreceptors. The sensitivity of these C mechanoreceptors varies within a wide range (Iggo, 1960). The threshold of the most sensitive units (hair 20 mg) was similar to that of the most sensitive A mechanoreceptors in the cat's skin (Witt & Hensel, 1959). A typical feature of the C mechanoreceptors was a marked after-discharge, as described first by Zotterman (1939). This phenomenon was never observed in the A fibres.

In the present investigation impulses of mechanosensitive C fibres have not been studied systematically. We have confirmed previous findings (Iggo, 1959*a*, *b*, 1960), that C mechanoreceptors were excited by cooling the skin. In our occasional observations the maximum frequencies of three different C fibres amounted to only 17, 11 and 5 impulses/sec on sudden severe cooling from 32 to 8° C, whereas mechanical stimulation caused a discharge frequency as high as 100 impulses/sec. The lowest threshold for a phasic discharge of a C mechanoreceptor was cooling by 2° C. The discharge of two C fibres is shown in Fig. 9; one was excited by heating the skin (a warm fibre) and the other (spikes downward) reacting to light mechanical vibration of the thermode due to turbulence caused by switching on the hot and cold water supply. The latter fibre was excited briefly during cooling of the skin, whereas the warm fibre was not.

TABLE 1. Responses of single thermosensitive C fibres

Fibre no.	Conduction velocity (m/sec)	Response to thermal stimulation†		Max. frequency (imp./sec)	Threshold temp. change (° C) (initial temp. *ca.* 32° C)	Temp. for steady discharge (° C)		Response to mechanical stimulation‡
		Cooling	Warming			Lowest	Highest	
1	1·1	+	−	38	< −1 (*x*)	14	26 (> 30)	0
2	*C**	+	−	45	< −4	.	< 28	0
3	1·5	+	−	55	−0·2	< 6·5	41	0
4	.	+	−	.	.	.	*ca.* 32	0
5	0·6	+	−	40	.	5	32	0
6	*C*	−	+	55	+0·3	> 36	.	Threshold 2 g
7	.	−	+	65	+0·3	38·5	43	0
8	.	.	+	.	39 (*xx*)	> 48	.	0
9	.	.	+	.	42	45	.	0
10	*C*	−	+	80§	41	41	57	0
11	.	.	+	60	.	*ca.* 40	.	0
12	.	.	+	44	44	46	.	Firm pressure
13	.	.	+	.	41	.	.	0
14	.	−	+	50§	37	38·5	.	0
15	0·85	.	+	.	43	.	.	High threshold
16	.	.	+	.	< 47	> 48	.	0
17	*C*	−	+	38§	38	42	.	0
18	.	−	+	.	38·5	.	.	0

(*x*) The final values of the external temperature change are given.

(*xx*) For fibres 8–18 the absolute values of the final temperature are given.

* *C* indicates that the conduction velocity was less than 2 m/sec, but was not measured accurately.

† +, Excitation; −, inhibition. The effect depended on the initial temperature; when this was in the range where the fibre was excited then the activity was inhibited, otherwise there was no response. ‡ 0 = no response. § Bursts.

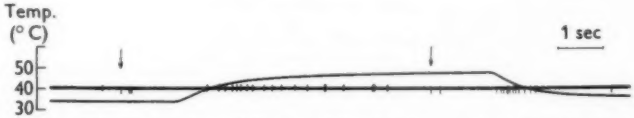


Fig. 9. Afferent impulses of two C fibres and the skin temperature when heating and cooling the skin. Arrows mark the slight mechanical vibration of the thermode caused by changing the water flow. There are two active fibres in this strand. The diphasic impulses are from a unit excited by heating the skin. This unit was not excited by the light mechanical stimuli which excited the other fibre. This latter fibre was excited by suddenly cooling the skin.

DISCUSSION

The old and controversial problem of 'specificity' of cutaneous sensory mechanisms has been carried to a new stage by recent electrophysiological and histological investigations. It should be kept in mind that the concept

of 'specific' sensory fibres or receptors has two quite different aspects: (1) the specific *sensation* aroused by stimulation of a receptor ('specific energy' in the sense of Müller (1844) and (2) the response of a *receptor* to a specific stimulus. Of course, only the second problem can be adequately solved by animal experiments, and even then it remains uncertain whether the results can be properly applied to man. Since certain cutaneous and other receptors in the cat respond to both mechanical and thermal stimulation (Hensel & Zotterman, 1951*b*; Hensel, 1952; Witt & Hensel, 1959; Iggo, 1959*a*, *b*, 1960; Douglas & Ritchie, 1959; Lele & Weddell, 1959), the classification of cutaneous receptors as 'mechanoreceptors' or 'thermoreceptors' may seem to be arbitrary. However, *quantitative* measurements of the sensitivity of some individual receptors to both kinds of stimuli have revealed systematic differences. *Qualitative* experiments alone are not sufficient and may sometimes be misleading. Until the properties of an individual unit have been examined quantitatively it would perhaps be better not to use the terms 'specific' and 'non-specific' and it would certainly be premature to argue about peripheral 'specificity'. At the least it is necessary to define exactly the properties of the 'specific' receptor being dealt with. For the cold (warm) receptors the following definition is proposed with respect to the discharge in the afferent fibre: (1) Frequency rise (fall) on sudden cooling, (2) no response on sudden warming (cooling), if the fibre is silent, or an inhibition of a resting discharge, (3) a steady discharge dependent on temperature, (4) no response to non-painful mechanical stimulation (or at least a considerably higher threshold than the most sensitive mechanoreceptors, cf. Fig. 7, Iggo, 1960), (5) thermal sensitivity comparable with temperature sense in man.

The results described in this paper demonstrate clearly the existence of cutaneous sensory C fibres whose endings are sensitive either to warming or to cooling, whereas they are not excited by mild or non-painful mechanical stimulation. Unlike the heat and cold fibres described by Iggo (1959*b*), which may be concerned with pain, several C fibres found in the present investigation were stimulated by temperatures which in man cause no pain at all. The thresholds of these cold and warm receptors, amounting to only a few tenths of a degree from an indifferent temperature (30–33° C), correspond well with the thresholds of cutaneous cold and warm sensation in man (Hensel, 1952). On our proposed definition they are thermoreceptors.

The general behaviour of the cutaneous C thermoreceptors is comparable with that of the thermoreceptors in the cat's tongue. The values for the dynamic and static responses of the non-myelinated cold and warm fibres (Table 2) are of the same order as those found for the specific cold receptors in the tongue (Hensel & Zotterman, 1951*a*). However, there are some dif-

ferences between the C thermoreceptors in the skin and the thermoreceptors in the tongue. The lingual nerve of the cat apparently contains no C fibres sensitive to mild thermal stimuli (Zotterman, personal communication); in this nerve there are A fibres sensitive to gentle warming or cooling. The cutaneous warm receptors with C fibres are more sensitive than the lingual warm fibres and show no paradoxical discharge (Dodt & Zotterman, 1952) during cooling. Further, the C cold fibres from the skin seem to be active at lower constant temperatures and to have a maximum discharge at a lower temperature than the cold fibres in the tongue.

TABLE 2. Maximum sensitivity (impulses/sec.° C) of single temperature fibres in the cat

	Cutaneous warm receptor with C fibre	Cutaneous cold receptor with C fibre	Lingual cold receptor with A fibre*
Dynamic sensitivity	+33	−30	−30
Static sensitivity: Positive range	+ 8	+ 3	+ 1
Static sensitivity: Negative range	− 5	− 0·7	− 2·5

* From Hensel & Zotterman (1951*a*).

A number of A fibres which responded to thermal as well as light mechanical stimulation were found by Witt & Hensel (1959) in the saphenous nerve of the cat. No fibres were detected in the A group which were stimulated by temperature alone. These findings support the view that cutaneous temperature impulses in the cat may be carried mainly in non-myelinated fibres. We do not know the cat's temperature sensation, but the existence of thermal receptors is indicated by vasomotor reflexes in the ear to moderate heating or cooling of the legs in the unanaesthetized cat. These reactions were not caused by changes in blood temperature, which were actually the reverse of those necessary to give the observed changes in ear blood flow. The recorded hypothalamic and rectal temperatures rose during cooling and fell during heating of the extremities (Kundt, Brück & Hensel, 1957).

There are several reports dealing with the excitation of cutaneous fibres by both mild mechanical stimuli and cooling. Some cutaneous A fibres (Witt & Hensel, 1959) and C fibres (Douglas *et al.* 1959; Iggo, 1959*a*, 1960; this paper) in the cat are excited by non-injurious pressure and by cooling. The A fibres sometimes gave a steady discharge with constant temperatures whereas the C fibres were excited only when the cutaneous temperature was falling. The results have been interpreted in different ways. Iggo (1960) has suggested that the C fibres innervate mechanoreceptors which, in common with other mechanoreceptors, are excited by cooling. Douglas *et al.* (1959) consider that the response may indicate dual specificity of the

endings. The present results, which show clearly that there are cutaneous C thermoreceptors which are much more sensitive to thermal changes than the C mechanoreceptors, provide a more convincing explanation for cutaneous thermosensitive mechanisms. If the A and C fibres described do indeed innervate mechanoreceptors, the results may account for the old observation (Weber's illusion, 1846) that weights feel heavier when they are cold (Hensel & Zotterman, 1951*b*; Douglas *et al.* 1959; Witt & Hensel, 1959).

In the last few years, in what has aptly been referred to as 'the tide of recent work', the classical doctrine of specific cutaneous receptors has been severely attacked on the basis of new histological findings and physiological investigations (Hagen, Knoche, Sinclair & Weddell, 1953; Weddell, Palmer & Pallie, 1955; Sinclair, 1955; Lele & Weddell, 1956, 1959; Oppenheimer, Palmer & Weddell, 1958; Weddell, Palmer & Taylor, 1959). Weddell *et al.* (1955) confirmed the original observation of Adrian (1931) that touching hairs sets up impulses in rapidly-conducting cutaneous fibres. Thermal stimuli failed to do so. Since the hair follicles have a distinctive innervation by large nerve fibres, Weddell (1960) now considers that their nerve endings may be specifically excited by light mechanical stimuli. To this extent he thus revives von Frey's (1895) suggestion that different qualities of cutaneous sensation are served by distinct histological structures. The other major type of afferent innervation of hairy skin is the diffuse arborization of 'free' nerve endings (Weddell *et al.* 1959). These presumably serve the other modalities (Weddell, 1960) even though there are apparently no histological differences to be seen among them. However, as Sinclair (1955) admits, it remains possible that specialized endings exist, but that the differences are too subtle to be revealed by present histological methods. This latter view is supported by the present work on C fibres innervating receptors in hairy skin and by recent investigations on the neurohistology of the cat's tongue, where Kantner (1957) has found only a network of 'free' nerve endings, although specific mechanoreceptors and thermoreceptors are present in the area. On the other hand there are reports of cutaneous nerve endings which, it is claimed, are not highly specific. In some regions, such as the cornea (Lele & Weddell, 1959) the non-specific fibres are thought to predominate. These conclusions rely on experiments with multi-fibre techniques for recording from the cutaneous nerves. Such techniques give results which are notoriously difficult to interpret. In addition, an important assumption in Lele & Weddell's argument appears to be that specific thermoreceptors are absent from the cornea. The present work makes this assumption less convincing. Lele & Weddell may have missed the thermoreceptors because the afferent fibres were non-myelinated. They recorded impulses in the myelinated fibres only.

SUMMARY

1. Impulses in slowly-conducting (C) afferent fibres were recorded in fine strands dissected from the saphenous nerves of cats anaesthetized with chloralose. The conduction velocities in some of the fibres ranged from 0·6 to 1·5 m/sec.

2. In response to well defined thermal stimuli the fibres responded either to warming or to cooling of the skin. The thresholds for mechanical stimulation were high.

3. Changes in skin temperature of 0·2° C or less were sufficient to excite the most sensitive cold and warm fibres.

4. The dynamic sensitivity of some fibres was 30 impulses/sec.° C, which is as great as both the sensitivity of the most sensitive cold fibres in the cat's tongue and the temperature sense in man.

5. Constant temperatures led to a steady discharge of impulses, with a maximum between 16 and 27° C for the cold fibres and above 41° C for the warm fibres.

The participation of A.I. in the experiments described in this paper was made possible by a grant from the 'Deutsche Forschungsgemeinschaft'.

REFERENCES

ADRIAN, E. D. (1931). The messages in sensory nerve fibres and their interpretation. *Proc. Roy. Soc.* B, **109**, 1–18.

DODT, E. & ZOTTERMAN, Y. (1952). Mode of action of warm receptors. *Acta physiol. scand.* **26**, 345–357.

DOUGLAS, W. W. & RITCHIE, J. M. (1959). The sensory functions of the non-myelinated afferent nerve fibres from the skin. *Ciba Foundation Study Group, No.* 1, *Pain and Itch*, pp. 26–40. London: Churchill.

DOUGLAS, W. W., RITCHIE, J. M. & STRAUB, R. W. (1959). Discharges in non-myelinated (C) fibres in the cat's saphenous nerve in response to changing the temperature of the skin. *J. Physiol.* **146**, 47–48*P*.

HAGEN, E., KNOCHE, H., SINCLAIR, D. C. & WEDDELL, G. (1953). The role of specialized nerve terminals in cutaneous sensibility. *Proc. Roy. Soc.* B, **141**, 279–287.

HENSEL, H. (1952). Physiologie der Thermoreception. *Ergebn. Physiol.* **47**, 166–368.

HENSEL, H. (1953). Ein Photokymographion für kombinierte Registrierung von Kathodenstrahloszillogrammen und Lichtzeigerausschlägen. *Pflüg. Arch. ges. Physiol.* **256**, 37–42.

HENSEL, H., IGGO, A. & WITT, I. (1959). Cutane Thermoreceptoren mit marklosen afferenten Fasern. *Pflüg. Arch. ges. Physiol.* **270**, 82–83.

HENSEL, H. & ZOTTERMAN, Y. (1951*a*). Quantitative Beziehungen zwischen der Entladung einzelner Kältefasern und der Temperatur. *Acta physiol. scand.* **23**, 291–319.

HENSEL, H. & ZOTTERMAN, Y. (1951*b*). The response of mechanoreceptors to thermal stimulation. *J. Physiol.* **115**, 16–24.

IGGO, A. (1958*a*). The electrophysiological identification of single nerve fibres, with particular reference to the slowest-conducting vagal afferent fibres in the cat. *J. Physiol.* **142**, 110–126.

IGGO, A. (1958*b*). Single C fibres from cutaneous receptors. *J. Physiol.* **143**, 47–48*P*.

IGGO, A. (1959*a*). A single unit analysis of cutaneous receptors with C afferent fibres. *Ciba Foundation Study Group, No.* 1, *Pain and Itch*. pp. 41–56. London: Churchill.

IGGO, A. (1959*b*). Cutaneous heat and cold receptors with slowly-conducting (C) afferent fibres. *Quart. J. exp. Physiol.* **44**, 362–370.

IGGO, A. (1960). Cutaneous mechanoreceptors with afferent C fibres. *J. Physiol.* **152**, 337–353.

KANTNER, M. (1957). Die Sensibilität der Katzenzunge. *Acta neuroveg., Wien*, **15**, 223–234.

KUNDT, H. W., BRÜCK, K. & HENSEL, H. (1957). Hypothalamustemperatur und Hautdurchblutung der nichtnarkotisierten Katze. *Pflüg. Arch. ges. Physiol.* **264**, 97–106.

LELE, P. P. & WEDDELL, G. (1956). The relationship between neurohistology and cutaneous sensibility. *Brain*, **79**, 119–154.

LELE, P. P. & WEDDELL, G. (1959). Sensory nerves in the cornea and cutaneous sensibility. *Exp. Neurol.* **1**, 334–359.

MÜLLER, J. (1844). *Handbuch der Physiologie des Menschen*. Vol. 1, p. 667ff. Coblenz: Hölscher.

OPPENHEIMER, D. R., PALMER, E. & WEDDELL, G. (1958). Nerve endings in the conjunctiva. *J. Anat., Lond.*, **92**, 321–352.

SINCLAIR, D. C. (1955). Cutaneous sensation and the doctrine of specific energy. *Brain*, **78**, 584–614.

VON FREY, M. (1895). Beiträge zur Sinnesphysiologie der Haut, III. *Ber. sächs. Ges. (Akad.) Wiss.* **47**, 166–184.

WEBER, E. H. (1846). Der Tastsinn und das Gemeingefühl. In Wagner's *Handwörterbuch der Physiologie*, Vol. III/2, pp. 481–588. Braunschweig: Vieweg.

WEDDELL, G. (1960). The structure of the skin in relation to its innervation. In *Progress in the Biological Sciences in Relation to Dermatology*, pp. 55–63. London: Cambridge University Press.

WEDDELL, G., PALMER, E. & PALLIE, W. (1955). Nerve endings in mammalian skin. *Biol. Rev.* **30**, 159–195.

WEDDELL, G., PALMER, E. & TAYLOR, D. (1959). The significance of the peripheral anatomical arrangements of the nerves which serve pain and itch. *Ciba Foundation Study Group, No.* 1, *Pain and Itch*, pp. 3–10. London: Churchill.

WITT, I. & HENSEL, H. (1959). Afferente Impulse aus der Extremitätenhaut der Katze bei thermischer und mechanischer Reizung. *Pflüg. Arch. ges. Physiol.* **268**, 582–596.

ZOTTERMAN, Y. (1939). Touch, pain and tickling: an electrophysiological investigation on cutaneous sensory nerves. *J. Physiol.* **95**, 1–28.

Note added in proof

I. Iriuchijima & Y. Zotterman (*Acta physiol. scand.*, in the Press) have also recorded from cutaneous thermal fibres similar to those described in this paper.

J. Physiol. (1960), **153**, *pp.* 127–130
Printed in Great Britain

THE ABSENCE OF POSITION SENSE IN THE HUMAN EYE

BY G. S. BRINDLEY AND P. A. MERTON
From the Physiological Laboratory, University of Cambridge

(*Received* 16 *March* 1960)

It has been widely held (e.g. Sherrington, 1918; Hoffmann, 1934, p. 28) that we have a sense of position of the eyeball which depends on afferent nerve fibres from the extraocular muscles. However, the experiments of Helmholtz (1867) indicate that such a position sense, if it exists, is not used to correct visual impressions when the eye is passively displaced. The following experiments, done on four subjects, show that the eye has no position sense.

EXPERIMENTS

Passive deviation of one eye causes no sensation. One conjunctival sac was anaesthetized with cocaine. With the subject lying supine, an opaque aluminium shell, shaped to fit the eye, was placed so that it occluded the whole of the cornea and a surrounding ring of sclera about 2 mm in width. The insertion of the lateral or medial rectus muscle was seized through the conjunctiva with fine-toothed forceps, and the eye passively abducted or adducted by amounts corresponding to up to 40° of visual angle, the other (unanaesthetized) eye remaining closed or covered by the subject's hand. The subject was found to be entirely unaware of the direction of any passive movement and, if care was taken to avoid accidental disturbance of the eyelids, he was unaware that it had been made, even for sudden movements of several tens of degrees.

Passive deviation of one eye causes no substantial reflex movement of the other. If during a passive movement of the occluded eye the other eye was kept open, the subject saw no apparent movement of things around him. This strongly suggests that passive deviation of one eye does not reflexly cause movement of the other, since sudden movements of the whole retinal image through as little as 5′ are easily detected. There is, however, the alternative possibility that reflex eye movements occur, but that the brain compensates for the resulting movement of the retinal image, so that it is not detected; such compensation would be analogous to that which is well known to occur during voluntary eye movements, but much more complete. Evidence to which this objection does not apply comes from the following experiment, which was suggested by

Mr R. L. Gregory, who also kindly lent us his fixation lantern. The subject lay in darkness except for a small red fixation light surrounded by a faint blue halo which subtended 1° at his eye. As long as the red light was accurately fixated, the halo was invisible, since it fell wholly on the fovea, which is relatively insensitive to blue light; but movement through 30′ sufficed to make it easily seen. One eye was anaesthetized and its cornea occluded. Passive movements of this eye while the other eye fixated the red light were found not to make the halo visible. We conclude that passive movement of one eye through as much as 40° certainly causes no reflex movements of the other exceeding 30′, and probably (on evidence of the lack of apparent movement of things seen) causes none exceeding 5′.

Simultaneous passive movements of both eyes. William James (1890) objected to certain observations on unilateral ocular palsies adduced by Helmholtz, on the grounds that no account was taken of sensory information from the sound eye. This objection was adopted by Sherrington (1900), and the later answer of Jackson & Paton (1909) apparently escaped notice. To forestall possible criticism of the present experiments on the same lines, both conjunctival sacs were anaesthetized, both corneae occluded with opaque shells, and both eyes passively deviated in the same direction at the same time. The subject was found to be unaware of deviations of up to 30° to right or left from the mid position.

Active movements. With both corneae occluded, the subject was asked to deviate the eyes to right or left. During some of these attempted movements the eyes were allowed to move freely through 30° or more. During others both eyes were held firmly with forceps, and could move through no more than about 5°. An assistant held the lids of both eyes widely open, both to prevent sensory clues from deformation of skin by the corneal caps when they moved, and to avoid displacement of the caps at extreme deviations. With satisfactory anaesthesia of the conjunctiva, it was found that the subject could not tell whether the eyes were held or not; he regularly had the impression that he succeeded in moving them through a large angle.

Subsidiary observations. In one subject both eyes (without caps on the corneae) were held with forceps while he attempted to deviate the eyes actively. Apparent movements of external objects in the direction of the attempted movement were seen. The subject thus interprets his visual impressions as if he had succeeded in moving his eyes. Similar observations were made by Mach (1886, p. 57), who restrained movements of his eyes to the right with 'two large lumps of moderately hard putty firmly pressed against the right side of each eye-ball', and by Kornmüller (1931), who paralysed the four rectus muscles of one eye by injecting novocaine into them. One of us (P.A.M.) performed a related experiment. He

received intravenously a dose of curare sufficient to cause weakness but not paralysis of his eye muscles. On voluntary deviation of the eyes he observed apparent movements of external objects in the direction of the deviation.

DISCUSSION

We find that, when the conjunctival sacs are anaesthetized and visual clues excluded, subjects know neither the amount nor the direction of any deviation of their eyes unless that deviation has been produced by the unhindered action of their own eye muscles. The errors that they make when the muscles are prevented from moving the eyes are just those that would be expected on Helmholtz's view that non-visual knowledge of the position of the eyes depends exclusively on judgement of the effort of will employed in attempting to move the eyes. They are very difficult to reconcile with the view that it depends on any known sense organs, especially muscle spindles, although muscle spindles are known to be present in the eye muscles of man (Buzzard, 1908; Cooper & Daniel, 1949) as in those of other primates (Tozer & Sherrington, 1910, at p. 451; Cooper & Daniel, 1949). Possible functions for muscle spindles are discussed by Hammond, Merton & Sutton (1956).

Meaning of the term 'position sense'. We use the term 'position sense' with the meaning that has long been customary in clinical neurology: a part of the body is said to have position sense if the subject knows its relation to the rest of the body by information derived from mechanically stimulated sense organs in it or anatomically connected to it. Thus the knowledge of the relation of his eyes to his head which a subject can gain by seeing his nose or moustache (Mach, 1886, p. 14) is not deemed to show that he has position sense in the eye, just as a tabetic patient is not said to have position sense in his toes if he can recognize their relation to his feet only by looking at them.

Influence of anaesthesia of the conjunctival sac. Though in our experiments the conjunctival sac was always anaesthetized, it is unlikely that this anaesthesia inactivated receptors which in the normal use of the eyes give much information about their relation to the head, for the accuracy with which subjects whose eye muscles are acting without hindrance can judge or reproduce positions of their eyes is not impaired by anaesthetizing the conjunctival sac (Merton, 1960).

SUMMARY

1. Subjects cannot detect passive movements of one eye or of both together if their conjunctival sacs are anaesthetized and visual clues excluded.

2. Under the same conditions they cannot tell whether attempts to move the eyes actively are successful or are mechanically prevented.

3. Passive movement of one eye causes no detectable reflex movement of the other.

REFERENCES

BUZZARD, E. F. (1908). A note on the occurrence of muscle-spindles in ocular muscles. *Proc. R. Soc. Med.* **1**, *Neurological Section*, 83–87.

COOPER, S. & DANIEL, P. M. (1949). Muscle spindles in human extrinsic eye muscle. *Brain*, **72**, 1–24.

HAMMOND, P. H., MERTON, P. A. & SUTTON, G. G. (1956). Nervous gradation of muscular contraction. *Brit. med. Bull.* **12**, 214–218.

HELMHOLTZ, H. VON (1867). *Handbuch der physiologischen Optik*, 1st ed., section 29, pp. 599–601. Leipzig: Voss. English translation by SOUTHALL, J. P. C. (1925). *Helmholtz's Treatise on Physiological Optics*, vol. 3, section 29, pp. 243–245. Menasha, Wisconsin: Optical Society of America.

HOFFMANN, P. (1934). Die physiologischen Eigenschaften der Eigenreflexe. *Ergebn. Physiol.* **36**, 15–108.

JACKSON, J. H. & PATON, L. (1909). On some abnormalities of ocular movements. *Lancet*, **176**, 900–905.

JAMES, W. (1890). *The Principles of Psychology*, vol. 2, p. 507. London: MacMillan.

KORNMÜLLER, A. E. (1931). Eine experimentelle Anästhesie der äusseren Augenmuskeln am Menschen und ihre Auswirkungen. *J. Psychol. Neurol., Lpz.*, **41**, 354–366.

MACH, E. (1886). *Beiträge zur Analyse der Empfindungen*, 1st ed. Jena: Fischer. English translation (1959). *The Analysis of Sensations*. New York: Dover; London: Constable.

MERTON, P. A. (1960). The accuracy of directing the eyes and the hand in the dark. *J. Physiol.* (in the Press).

SHERRINGTON, C. S. (1900). The muscular sense. In SCHÄFER, E. A., *Textbook of Physiology*, p. 1004. Edinburgh and London: Pentland.

SHERRINGTON, C. S. (1918). Observations on the sensual rôle of the proprioceptive nerve-supply of the extrinsic ocular muscles. *Brain*, **41**, 332–343.

TOZER, F. M. & SHERRINGTON, C. S. (1910). Receptors and afferents of the third, fourth and sixth cranial nerves. *Proc. Roy. Soc.* B, **82**, 450–457.

J. Physiol. (1960), **153**, *pp.* 131–145
With 7 text-figures
Printed in Great Britain

ACUTE INSULIN DEFICIENCY PROVOKED BY SINGLE INJECTIONS OF ANTI-INSULIN SERUM

By J. ARMIN, R. T. GRANT and P. H. WRIGHT

From the Departments of Experimental Medicine and Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

(*Received* 21 *March* 1960)

After prolonged treatment with insulin, guinea-pigs and some other animals are known to yield sera which will abolish the hypoglycaemic effects of insulin injected simultaneously into mice (Moloney & Coval, 1955; Arquilla & Stavitsky, 1956; Moloney & Goldsmith, 1957; Moloney & Aprile, 1959), and the stimulant action of the hormone upon glucose consumption by the isolated rat diaphragm (Wright, 1959*a*). These effects are thought to be due to the presence of insulin antibodies which combine with the hormone and so prevent its action. The antibodies may also neutralize the effects of endogenously secreted insulin, for such serum is known to induce hyperglycaemia in mice (Moloney & Coval, 1955; Moloney & Goldsmith, 1957). The observations now reported strongly suggest that the serum of insulin-treated guinea-pigs will neutralize the effects of endogenously secreted insulin in the rabbit, rat and cat, but not in the guinea-pig itself; a preliminary account has been published (Wright, 1959*b*).

METHODS

Production of anti-insulin serum. Recrystallized bovine insulin (10 mg, 22·2 u./mg; Boots Pure Drug Co. Ltd.) dissolved in 10 ml. aqueous phenol 0·3 % (w/v) acidified with HCl to pH 2·6 approx., was mixed with liquid paraffin B.P. 7 ml. and anhydrous lanolin (*adeps lanae* B.P.) 3 ml. and the mixture emulsified in a Waring blender.

Groups of 5–8 albino guinea-pigs were injected subcutaneously at monthly intervals with 2 ml. of this thick emulsion, 1 ml. being injected between the shoulders and 0·5 ml. into the inner aspect of each thigh. Two weeks after the third and each subsequent monthly injection of antigen about 10 ml. of blood was withdrawn from each animal under light ether anaesthesia by cardiac puncture. The serum specimens from animals in each group were pooled and kept frozen (− 10° C) until required; pooled sera from different groups of animals were kept separate.

No hypoglycaemic reactions were observed at any time after the injections of insulin antigen. Most animals remained in good health for periods of 6–17 months. The majority of deaths occurred immediately after cardiac puncture and were due to haemorrhage into the pericardial or pleural cavities; a few animals became thin and died for no apparent reason.

In the following description, serum obtained in this way is termed anti-insulin serum. Serum from guinea-pigs treated for 3 months under similar conditions with the same

emulsion, but containing no added insulin, is termed control serum. Normal untreated guinea-pigs were used to obtain normal serum.

Experimental animals. Serum was injected intravascularly in single doses into conscious rats and rabbits and into anaesthetized rats, rabbits, a guinea-pig and a cat; Professor J. M. Robson of the Pharmacology Department of this school carried out the experiment on the cat. All these animals were fed on standard diets. When necessary, food, but not water, was withdrawn 24–48 hr beforehand.

The rats, each weighing 200–300 g, were albino animals of a Wistar strain originally supplied by the Chester Beatty Research Institute and bred at this school. The rabbits, with half to three-quarter lop ears, usually weighed 2–3 kg. One ear was deprived of its sympathetic nerves by excising the ipsilateral superior cervical and stellate ganglia at least 1 week beforehand; the vessels of such denervated ears provide a sensitive indicator for the presence of vasoconstrictor substances in the blood stream. Adrenalectomy of the rabbit was performed by a one-stage operation, the animal being subsequently maintained in good health by injections of cortisone or DOCA (deoxycortone acetate B.P.) (Armin & Grant, 1959).

Anaesthesia was induced with pentobarbitone sodium (30 mg/kg = 0·5 ml./kg veterinary Nembutal; Abbott Laboratories Ltd.) and maintained by smaller doses repeated as required. Local procaine 2% (w/v) anaesthesia was also used during the introduction of catheters. For intravascular catheterization of the rat and guinea-pig, fine polythene tubing (internal diameter 0·011 mm; size PE 10; Clay Adams Co. Inc., New York) was used; larger tubing (internal diameter 0·023 mm; size PE 50) was preferred for the rabbit and cat. Such catheters were usually inserted into the femoral artery or vein; in the conscious rabbit into the marginal vein or central artery of the ear. Patency was maintained by the slow infusion of NaCl solution 0·9% (w/v) from a perfusion pump. A Perspex three-way tap (Armin & Grant, 1953) inserted in the length of the catheter allowed the injection of serum, withdrawal of blood samples and measurement of blood pressure by capacitance or mercury manometer. In the rabbit the temperature of the sympathectomized ear was measured by thermocouple.

Conscious rabbits were restrained in the box already described (Armin & Grant, 1957). In conscious rats, serum in doses of up to 1 ml. was injected into a tail vein by a technique similar to that described by Lazarow & Palay (1946). To keep the tail veins dilated the rats were kept in a warm cage, and just before injection the tails were immersed in warm water (40–50° C). Blood samples were obtained from the severed end of the rat's tail and collected on watch-glasses containing dried anticoagulant solution (1 drop 3·0% potassium oxalate and 1 drop 0·3% potassium fluoride). For serum injection and blood sampling conscious rats were restrained in boxes from which the tail protruded. The blood sugar concentration was determined on 0·2 ml. samples by a modification (Wright, 1957) of the method described by King (1951).

RESULTS

Hyperglycaemia induced by anti-insulin serum

The anti-insulin serum injected into rats, rabbits and the cat provoked a transient hyperglycaemia in each instance, but in the guinea-pig did not alter the blood-sugar concentration. No significant change in blood-sugar concentration followed the injection of normal or control serum into any of these animals.

Guinea-pig. Into one anaesthetized animal 1 ml. normal serum was injected by way of the femoral artery, and this was followed half an hour later by 1 ml. anti-insulin serum (batch X). No appreciable change in blood-sugar concentration was found in samples drawn at 5 min intervals

from the time of the normal serum injection up to 30 min after injection of the anti-insulin serum.

Cat. The intra-arterial injection of 3 ml. normal serum into the anaesthetized cat quickly provoked a strong but transient reaction (presumably due to the foreign protein), shown by sneezing, coughing, dyspnoea and cyanosis, together with a sharp rise of blood pressure and heart rate;

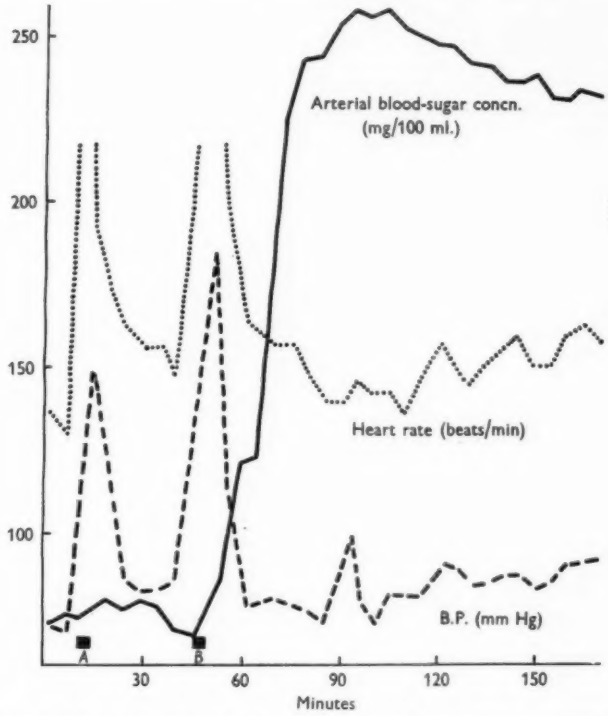


Fig. 1. Cat, 3 kg, anaesthetized. Records of arterial blood-sugar concentration, mean arterial blood pressure and heart rate, before and after intra-arterial (femoral) injection at *A* of normal guinea-pig serum (3 ml.) and at *B* of anti-insulin serum (3 ml.; batch X).

a similar reaction followed the injection 35 min later of 3 ml. anti-insulin serum (batch X). As Fig. 1 shows, however, no significant change in blood-sugar concentration followed the first injection, whereas following injection of the anti-insulin serum the level rose steeply to a maximum of about 250 mg/100 ml. in 45 min and was still high (230 mg/100 ml.) when the experiment was ended after 2 hr.

Rabbit. The intravascular injection of normal or anti-insulin serum in

doses of 1 ml./kg body weight into conscious or anaesthetized animals provoked no obvious reaction. Mean blood pressure and heart rate remained unchanged, the temperature of the sympathectomized ear did not fall and the ear vessels remained dilated. No appreciable rise in blood-sugar concentration followed injections of normal serum but anti-insulin serum evoked a conspicuous increase, detectable within 5 min. In five rabbits the blood sugar concentrations rose initially at rates ranging from 2·4 to 3·7 mg/100 ml./min (mean 3·04) and reached maxima of 200–300 mg/100 ml.; hyperglycaemia persisted for 3–5 hr. Figures 2 and 3 illustrate these results. Bilateral adrenalectomy with subsequent maintenance on cortisone or DOCA did not significantly alter the degree or duration of the response to anti-insulin serum injection.

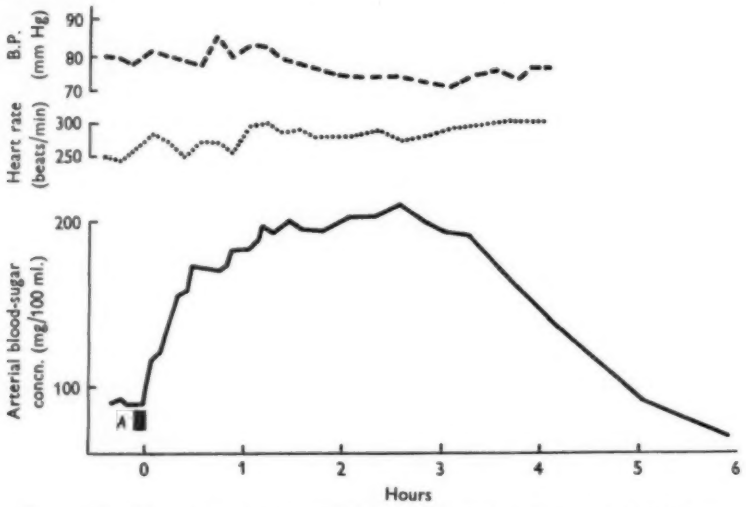


Fig. 2. Rabbit, 2·5 kg, fed, anaesthetized. Records of arterial blood-sugar concentration, mean arterial blood pressure and heart rate before and after intra-arterial (femoral) injection at *A* of 2·5 ml. guinea-pig anti-insulin serum (batch X).

When the injection of anti-insulin serum was repeated once or more at intervals of about a week, a strong foreign-protein reaction was provoked in both normal and adrenalectomized rabbits, whether conscious or anaesthetized. During this reaction, which lasted about half an hour, mean blood pressure and heart rate rose, the temperature of the sympathectomized ear fell and its vessels became constricted; the conscious animal became slightly restless and sneezed. The hyperglycaemic response, however, was similar to that of the normal animal receiving its first dose

(see Fig. 3). In one instance (Fig. 4), the fourth such dose of anti-insulin serum was preceded by an injection of normal serum. This normal serum provoked a strong foreign-protein reaction, including vasoconstriction in the sympathectomized ear, but only a slight transient increase in blood-sugar concentration. Anti-insulin serum injected half an hour later caused no such general reaction, but more than doubled the blood-sugar concentration.

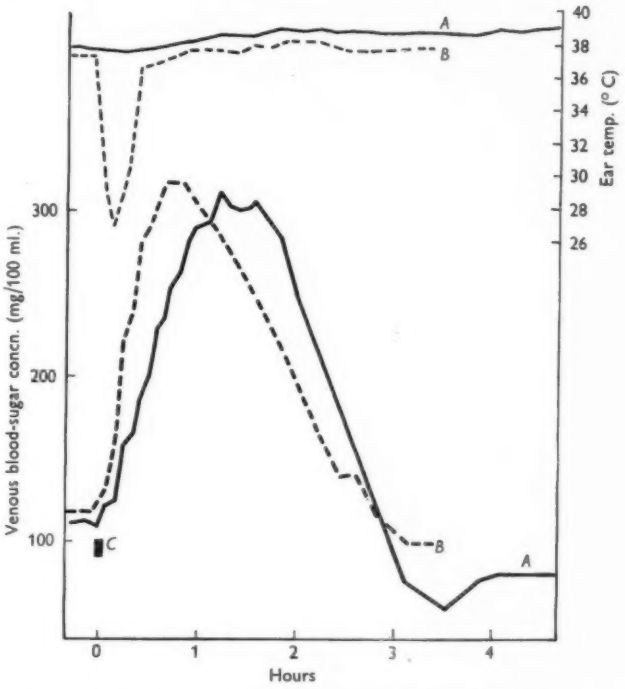


Fig. 3. Rabbit, 2·7 kg, fed, conscious; ear sympathectomized 2 months before first observations. Records of venous blood-sugar concentration (left-hand ordinate), and sympathectomized ear temperature (thermal junction, right-hand ordinate) before and after intravenous (marginal ear) injection at *C* of guinea-pig anti-insulin serum (1 ml./kg; batch X). Curves *A* show responses to 1st dose (2·7 ml.) and curves *B* show responses to 5th dose (2·8 ml.) 42 days later.

Rat. Normal, control and anti-insulin sera provoked no obvious foreign-protein reactions. No change in arterial blood pressure, pulse or respiration rates followed injection of control or anti-insulin serum (0·5 ml.) into an anaesthetized normal animal. Following normal or control serum the blood-sugar concentration fluctuated slightly and irregularly about the

pre-injection level, but showed no sustained change. Thus the maximum blood-sugar concentration observed in normal, fed, conscious rats after injection with control serum (four rats) or normal serum (three rats) was no more than 28 mg/100 ml. above the pre-injection level.

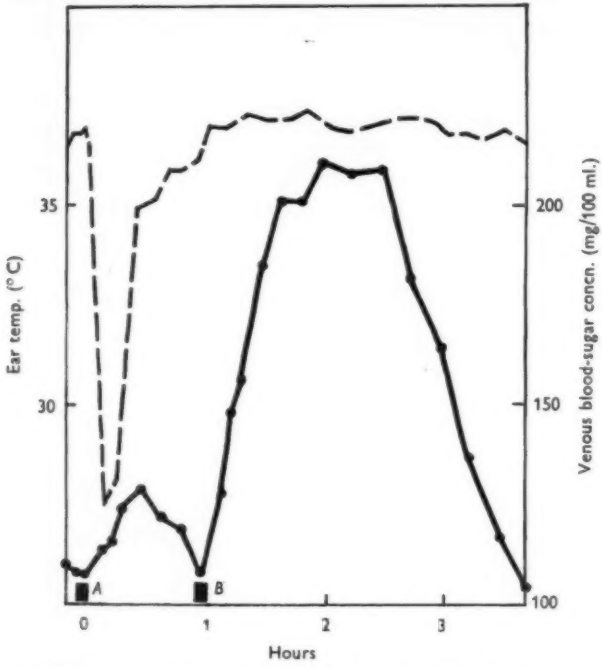


Fig. 4. Rabbit (same as in Fig. 3). Records of venous blood sugar concentration and sympathectomized ear temperature before and after intravenous (marginal ear) injection at *A* of normal guinea-pig serum (1 ml./kg) and at *B* of fourth dose of anti-insulin serum (1 ml./kg; batch X), 33, 19 and 5 days after 1st, 2nd and 3rd doses respectively of the anti-insulin serum.

Intravascular injection of anti-insulin serum (0·25–1·0 ml.) caused a marked but transient hyperglycaemia which was of about the same degree in both anaesthetized and conscious fed animals, either male or female. As Fig. 5 shows, after equal doses of anti-insulin serum the degree and duration of hyperglycaemia varied considerably in different animals though the initial rates of increase and subsequent maximum rates of fall in blood-sugar concentration were about the same. In eleven such experiments in which forty-four conscious, fed rats (180–220 g) were injected intravenously in groups of four with differing doses of six batches of anti-insulin serum, the mean rates of increase in blood-sugar concentration

during the first, second and third 15 min ($3{\cdot}31 \pm 0{\cdot}13$; $3{\cdot}12 \pm 0{\cdot}20$; $3{\cdot}13 \pm 0{\cdot}17$ mg/100 ml./min, respectively) were not significantly different from one another or from the mean rate measured over the whole period, namely $3{\cdot}16 \pm 0{\cdot}11$ mg/100 ml./min. Calculated from the maximum falls subsequently noted in 15 min in each of these animals, the mean maximum rate of fall in blood-sugar concentration ($5{\cdot}36 \pm 0{\cdot}24$ mg/100 ml./min) was significantly greater ($P < 0{\cdot}01$). In some animals a secondary rise in blood-sugar concentration occurred before or sometimes after the pre-injection level was regained.

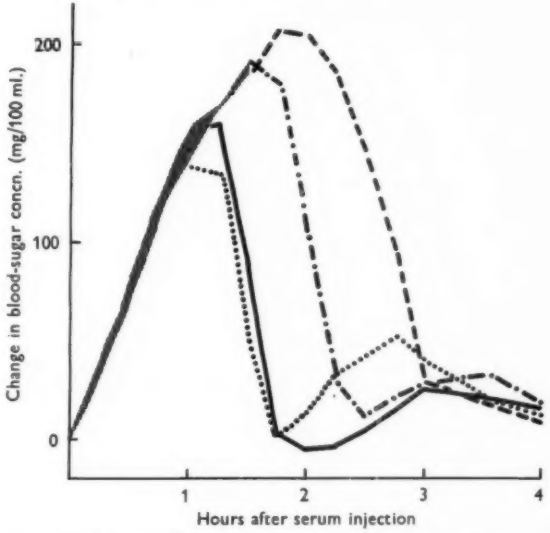


Fig. 5. Rats, female, conscious, fed. Changes in blood-sugar concentration in four animals (195–204 g) following injection into the tail veins of guinea-pig anti-insulin serum (1 ml.; batch Y).

Increasing doses of injected anti-insulin serum prolonged the period of hyperglycaemia and raised the maximum blood-sugar concentration induced. Figure 6 exemplifies the effects produced by three doses of one batch of anti-insulin serum in three groups each of four fed and conscious rats. The mean blood-sugar concentrations rose and subsequently fell at the same rates in each group but the maximum levels reached, and hence the periods of hyperglycaemia, increased with the dose injected.

A period of fasting before injecting anti-insulin serum reduces the degree but increases the duration of hyperglycaemia. Figure 7 shows the effect of fasting upon the response of female rats from the same litters to a dose of anti-insulin serum; male rats responded in the same way. In three

groups each of eight rats (four male and four female) the mean rate of rise in blood-sugar concentration in the first 45 min was significantly less ($P < 0.01$) in the group starved for 24 hr (1.38 ± 0.08 mg/100 ml./min) than in the fed animals (3.22 ± 0.15 mg/100 ml./min); in the group starved for 48 hr (1.76 ± 0.15 mg/100 ml./min) it was slightly greater ($P < 0.05$) than that in the group starved for 24 hr.

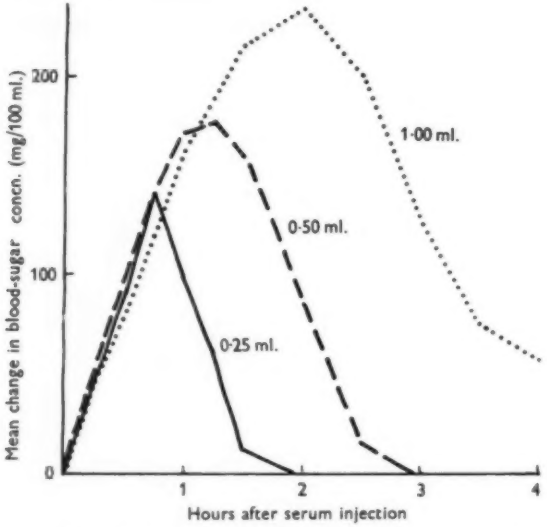


Fig. 6. Rats, female, conscious, fed. Mean changes in blood-sugar concentration in three groups of four animals of similar weights (178–218 g) following injection into the tail veins of 0·25, 0·50 and 1·0 ml. of guinea-pig anti-insulin serum (batch 6).

Anaesthesia has no appreciable effect upon the response of fed rats but reduces and prolongs the hyperglycaemic response of starved animals. Thus two rats deprived of food for 24 hr and then anaesthetized were each injected with 0·5 ml. of a batch of anti-insulin serum which provoked a conspicuous hyperglycaemia in fed, anaesthetized animals. In both rats the blood-sugar level rose steadily but at the very slow rates of 0·24 and 0·27 mg/100 ml./min for the period of 2 hr during which regular samples were taken.

When anti-insulin serum (2 ml./kg; batch 34943) was repeatedly injected intravenously into four normal male conscious rats 6, 19 and 27 days after the first dose, the hyperglycaemic response to the last was greater than those observed previously. Control serum (batch 48) injected in the same dose 34 days after the first serum injection increased the blood-sugar concentrations by 40–50 mg/100 ml. in 30–45 min. One of these animals

was therefore injected under anaesthesia 7 days later, first with control serum (0·5 ml.) and then 1 hr later with anti-insulin serum (0·5 ml.). The control serum produced an immediate transient general reaction, shown by a fall in blood pressure and pulse rate, laboured respirations and a marked increase in blood-sugar concentration to 220 mg/100 ml. in 30 min. Subsequent anti-insulin serum injection, however, caused only a rise in blood-sugar concentration.

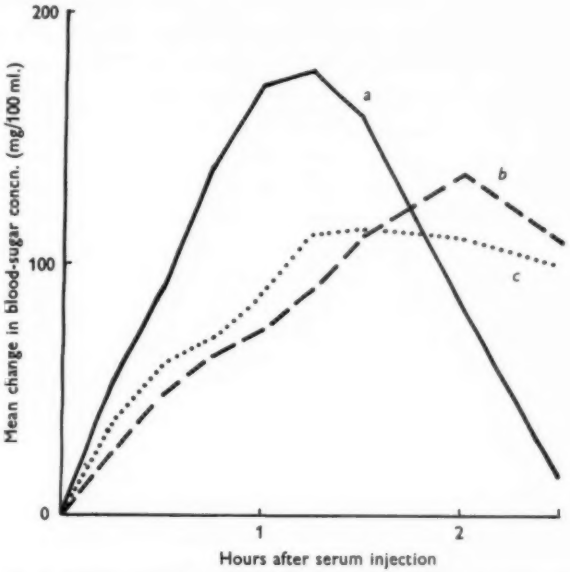


Fig. 7. Rats, female, conscious. Mean changes in blood-sugar concentration in three groups each of four animals of equal weights (210–218 g) following injection into the tail vein of anti-insulin serum (0·5 ml; batch 6) in the fed state (*a*) and after 24 hr (*b*) and 48 hr (*c*) fasting.

Assay of anti-insulin activity

One specimen of anti-insulin serum was kindly assayed for us by Messrs Boots Pure Drug Co. Ltd., by the mouse convulsion method; they assayed the residual insulin activity after mixing serum (2 ml.) with an excess (3 u.) of recrystallized bovine insulin. Of this sample (batch Y), 1 ml. neutralized the effects of 1·3 u. of insulin. The hyperglycaemic effects of graded doses (0·25, 0·50 and 1·00 ml.) of this and other batches of anti-insulin serum in groups of four conscious, fed rats were compared. Table 1 shows the batches of serum used, the doses injected and the areas under the response curves produced in each rat. Figure 5 illustrates the individual

responses produced in a typical experiment. The areas, A, were calculated by using the equation

$$A = \Sigma \Delta BS \times 15 \text{ mg/100 ml.} \times \text{min},$$

where ΔBS is the difference between the blood-sugar concentration at each 15 min interval from the time of serum injection to the time at which the concentration returns to the pre-injection level (or begins to rise again) and the pre-injection level itself.

TABLE 1. Areas under dose–response curves of female, fed, conscious rats injected intravenously with anti-insulin serum

Serum batch	Dose (ml.)	Area ($A \times 10^{-4}$)*					Anti-insulin potency (u./ml.)
		Rat 1	Rat 2	Rat 3	Rat 4	Mean	
Y	0·25	0·45	0·31	0·27	0·43	0·37	1·3
	0·50	0·93	0·70	0·84	0·84	0·83	
	1·00	0·98	2·45	0·84	1·59	1·45	
	0·77 + 0·5 u.*	0·51	0·58	0·62	0·47	0·54	
6	0·25	0·56	0·90	0·84	0·52	0·71	2·5
	0·50	1·25	0·91	1·58	2·58	1·58	
	1·00	2·60	4·41	3·10	3·14	3·31	
16204	0·25	0·86	0·57	0·56	0·91	0·73	2·3
	0·50	1·74	1·56	1·00	1·52	1·46	
14108	0·25	1·34	1·03	0·72	1·48	1·14	3·4
	0·50	2·86	0·96	2·08	2·14	2·01	
227328	0·50	1·32	0·94	1·76	1·24	1·32	2·1
424	0·50	1·50	1·03	0·95	0·89	1·09	1·8

* See text.

The area thus calculated is directly related to the dose of anti-insulin serum injected. For serum batch Y, for example, the relation between area and volume injected is given by the formula

$$A = 0{\cdot}89 + 1{\cdot}42\ (D - 0{\cdot}58),$$

where D is the volume (ml.) of serum injected.

Since 1 ml. of this serum contains sufficient antibody to neutralize 1·3 u. insulin, 0·5 u. should neutralize half the antibody in 0·77 ml. serum and leave unaffected antibody sufficient to produce a response equivalent to an area of 0·62. As is shown in Table 1, the mean area under the curves produced in four rats by this mixture was 0·54, a sufficiently good approximation to the estimated figure for our purpose. This example suffices to show that this method may be used to determine approximately the insulin-neutralizing potencies of the sera. Assuming that the area under a response curve is directly proportional to the insulin-neutralizing potency of the injected serum and that inactive serum produces no effect, the anti-insulin activity of the other batches was calculated from the results shown in Table 1.

Disappearance of insulin from the blood

An attempt was made to demonstrate the disappearance of insulin from the blood of an injected rabbit by using the isolated rat-diaphragm method of plasma insulin assay (Wright, 1957). The insulin-like activity of undiluted plasma obtained before was slightly greater than that of plasma drawn 45 min after injection of anti-insulin serum. It was thought, however, that the difference was probably insignificant. The matter was not pursued further because of the relative inaccuracy of this method of assay and also because of the difficulty of securing an adequate sample of blood for testing (25 ml.) without at the same time provoking adrenaline release, as shown by vasoconstriction in the sympathectomized ear.

DISCUSSION

In view of previous work by others (for references see Wright, 1960) and especially that of Moloney and his co-workers (Moloney & Coval, 1955; Moloney & Goldsmith, 1957; Moloney & Aprile, 1959) it seems reasonable to conclude that the transient hyperglycaemia provoked in the rat, cat and rabbit by the injection of guinea-pig anti-insulin serum is due to the neutralization of their endogenously secreted hormone by insulin antibodies which are not species-specific. Although we have failed to demonstrate the disappearance of insulin from the blood, one of us (Wright, 1959*a*) has shown that, *in vitro*, serum from some of the insulin-treated guinea-pigs used in the present experiments will abolish the stimulant effect of various insulins upon glucose consumption by the isolated rat diaphragm, but not that of synthetic hypoglycaemic agents such as synthalin and phenethyldiguanide. Further, it is clear that in normal animals the hyperglycaemia is not due to the introduction of a foreign protein or to release of endogenous adrenaline. In the normal cat, for example, a general reaction was produced by both normal and anti-insulin serum, but hyperglycaemia followed injection only of the latter. In the normal rabbit and rat neither normal nor anti-insulin serum provoked a general reaction and no vasoconstriction suggesting adrenaline release was observed in the sympathectomized ear of the rabbit. In both normal and adrenalectomized rabbits anti-insulin serum, but not normal serum, induced a conspicuous hyperglycaemia.

A feature of interest from the immunological point of view is that the intensity of the hyperglycaemic response in rabbits is not diminished by repeated injections of anti-insulin serum; thus in Fig. 3 the response to the 5th dose (curve *B*) does not differ significantly from that to the first (curve *A*). The response to the first injection could be due entirely to the neutralization of endogenous insulin by the antibody, but later responses

could be partly due to foreign-protein reactions which are apparent in the sensitized animal. In Fig. 4 it is seen, however, that the injection of normal serum into the same sensitized animal referred to in Fig. 3 produces a foreign-protein reaction but only very mild hyperglycaemia, whilst anti-insulin serum injected soon after evokes no foreign-protein reaction but hyperglycaemia which, though still conspicuous, is less than that found in previous or subsequent experiments in which anti-insulin serum alone was injected. In the normal rat repeated injections of anti-insulin serum resulted in an increase in the hyperglycaemic response and control serum produced marked hyperglycaemia associated with signs of a non-specific foreign-protein reaction in sensitized animals. The implications of these observations are not known and this matter has not been pursued further. It does, however, seem certain that endogenous release of adrenaline plays no significant part in the hyperglycaemic response induced in rabbits by anti-insulin serum, whether it is given for the first time or to an already sensitized animal.

The failure of the anti-insulin serum to provoke a hyperglycaemia in the guinea-pig was not unexpected. Moloney & Coval (1955) have shown that serum from guinea-pigs treated with bovine or pig insulins will neutralize the actions in mice of bovine, pig, sheep and rabbit insulins, but not that of insulin from the guinea-pig itself. Such insulin-treated guinea-pigs remain normoglycaemic and tolerate convulsive doses of pig insulin. They concluded, therefore, that the guinea-pig secretes insulin which is immunologically distinct from the hormones secreted by pigs, sheep, rabbits and cattle. Harris, Sanger & Naughton (1956) have found that bovine, pig, sheep and horse insulins differ slightly from one another in chemical composition, whilst minor immunological differences have been demonstrated by Berson & Yalow (1959). If, as seems likely, chemical structure is related to immunological behaviour, then the structure of guinea-pig insulin must differ markedly from that of any of the insulins mentioned above. Indeed, Goldsmith & Moloney (1957) have already shown that guinea-pig insulin possesses at least one physical property which distinguishes it from bovine insulin. It would be of interest, therefore, to extend the range of animals injected with guinea-pig anti-insulin serum; those which fail to become hyperglycaemic might also secrete insulin which differs in composition from those which have been investigated so far.

The estimates of insulin-neutralizing activity are admittedly only approximate but they have proved a useful guide for further work. They are higher than those obtained with similarly treated guinea-pigs by Moloney & Goldsmith (1957), whose titres generally ranged from 0·23 to 1·04 u. insulin/ml. serum and were only occasionally higher. The guinea-pigs from which all but one of the present batches (No. 16204) were ob-

tained yielded consistently more active serum than that of other guinea-pigs treated in the same way. It does appear, however, that the guinea-pig is capable of producing more potent anti-insulin serum than other animals. As such it proved very useful for our purposes, but since it is a small animal whose reaction to insulin as an antigen is very variable (Moloney & Goldsmith, 1957) it is not convenient for the production of large amounts of consistently potent serum.

The uniform rapid rise in blood-sugar concentration that begins so quickly after the injection of anti-insulin serum points to a possible explanation for the hyperglycaemia. The initial mean rate of increase in blood-glucose concentration in fed rabbits (3·04 mg/100 ml./min) is not significantly different from that found in a much larger group of fed rats ($3{\cdot}16 \pm 0{\cdot}11$ mg/100 ml./min); the sugar contents of bloods examined by the present method and by the specific glucose oxidase method were identical. From this the rate at which glucose enters the extracellular space may be calculated by assuming that this space is equivalent to 20% of the total body weight (Gamble, 1947) and that the concentration of glucose in the plasma, and hence in the extracellular space, is in the rabbit 1·5 times (Armin & Grant, 1959) and in the rat 1·3 times (personal observations in ten normal rats) that of the whole-blood concentration. On this basis glucose enters the extracellular space of the rabbit at a rate of 0·91 mg/min/100 g body weight, which is slightly greater than that in the rat (0·82 mg/min/100 g body weight). In the fasting rat the rate of glucose accumulation (0·36 mg/min/100 g body weight) is slower. In fed animals glucose enters the blood stream from the intestines, and the difference between the rates of glucose accumulation in fed and fasting rats may therefore represent the rate of glucose absorption from the gut (0·46 mg/min/100 g body weight). The liver is the major, if not the only, other source of glucose in the blood and it is still very doubtful whether insulin exerts any effect upon its metabolism. On the other hand, insulin does affect metabolism in extra-hepatic tissues, of which muscle and fat are the most important (see Stadie, 1954). It seems reasonable to conclude, therefore, that the glucose accumulating in the fasting injected rat represents the glucose normally consumed by extra-hepatic tissues under the influence of endogenously secreted insulin. If so, this rate is about a quarter of the total glucose turnover rate of normal rats, namely 1·67 mg/min/100 g body weight according to Feller, Strisower & Chaikoff (1950). The latter rate, however, includes the glucose consumption of the liver and also of the brain, which is not influenced by insulin. This hypothesis provides a working explanation for the hyperglycaemic effect of anti-insulin serum, but further work is required for proof.

SUMMARY

1. Serum from guinea-pigs treated with repeated subcutaneous injections of bovine insulin was injected intravascularly into rabbits, rats, a cat and a guinea-pig.

2. The transient hyperglycaemia induced in all animals except the guinea-pig is thought to be due to neutralization by insulin antibodies in the serum of endogenously secreted insulin; it is not due to non-specific foreign-protein reaction.

3. The degree and duration of hyperglycaemia produced in the rat is related to the dose of intravenously injected anti-insulin serum; a rough method of assay of insulin-neutralizing potency is described.

4. The rapid initial rate of increase in blood-sugar concentration observed in fasting rats probably reflects the normal rate of glucose consumption by insulin-sensitive tissues.

We wish to acknowledge the generous help of Messrs Boots Pure Drug Co. Ltd., who provided the recrystallized bovine insulin and especially of Mr K. L. Smith and Mr V. J. Birkinshaw who assayed the anti-insulin potency of serum Batch Y. We also wish to thank the Medical Research Council for a grant and Miss Sheila Haizelden and Mr Kenneth Kilbourn for technical assistance.

REFERENCES

ARMIN, J. & GRANT, R. T. (1953). The artery of the denervated rabbit's ear as a sensitive pharmacological test object. *J. Physiol.* **121**, 593–602.

ARMIN, J. & GRANT, R. T. (1957). The vasoconstriction caused by a pyrogen. *J. Physiol.* **138**, 417–433.

ARMIN, J. & GRANT, R. T. (1959). Adrenaline release during insulin hypoglycaemia in the rabbit. *J. Physiol.* **149**, 228–249.

ARQUILLA, A. R. & STAVITSKY, A. B. (1956). Evidence of the insulin-directed specificity of rabbit anti-insulin serum. *J. clin. Invest.* **35**, 467–474.

BERSON, S. A. & YALOW, R. S. (1959). Species specificity of human anti-beef, pork insulin serum. *J. clin. Invest.* **38**, 2017–2025.

FELLER, D. D., STRISOWER, E. H. & CHAIKOFF, I. L. (1950). Turnover and oxidation of body glucose in normal and alloxan-diabetic rats. *J. biol. Chem.* **187**, 571–588.

GAMBLE, J. L. (1947). *Chemical Anatomy, Physiology and Pathology of Extra-cellular Fluid.* Cambridge, Mass.: Harvard University Press.

GOLDSMITH, L. &. MOLONEY, P. J. (1957). Chromatography of guinea-pig native insulin. *Biochem. J.* **66**. 432–434.

HARRIS, J. I., SANGER, F. & NAUGHTON, M. A. (1956). Species differences in insulin. *Arch. Biochem. Biophys.* **65**, 427–438.

KING, E. J. (1951). *Microanalysis in Medical Biochemistry*, 2nd ed. London: Churchill.

LAZAROW, A. & PALAY, S. L. (1946). The production and course of alloxan-diabetes in the rat. *J. Lab. clin. Med.* **31**, 1004–1015.

MOLONEY, P. J. & APRILE, M. A. (1959). On the antigenicity of insulin; flocculation of insulin–antiinsulin. *Canad. J. Biochem. Physiol* **37**, 793–800.

MOLONEY, P. J. & COVAL, M. (1955). Antigenicity of insulin; diabetes induced by specific antibodies. *Biochem. J.* **59**, 179–185.

MOLONEY, P. J. & GOLDSMITH, L. (1957). On the antigenicity of insulin. *Canad. J. Biochem. Physiol.* **35**, 79–93.

STADIE, W. C. (1954). Current concepts of action of insulin. *Physiol. Rev.* **34**, 52–100.

WRIGHT, P. H. (1957). Plasma insulin estimation by the rat diaphragm method. *Lancet*, **273**, 621–624.

WRIGHT, P. H. (1959*a*). The effect of insulin antibodies on glucose uptake by the isolated rat diaphragm. *Biochem. J.* **71**, 633–638.

WRIGHT, P. H. (1959*b*). Production of acute insulin deficiency by administration of insulin antiserum. *Nature, Lond.*, **183**, 829–830.

WRIGHT, P. H. (1960). Insulin antibodies. *Brit. med. Bull.* (in the Press).

J. Physiol. (1960), **153**, *pp.* 146–165
With 1 *plate and* 5 *text-figures*
Printed in Great Britain

EXPERIMENTAL DIABETES IN RATS PRODUCED BY PARENTERAL ADMINISTRATION OF ANTI-INSULIN SERUM

By J. ARMIN, R. T. GRANT and P. H. WRIGHT

From the Departments of Experimental Medicine and Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

(*Received* 21 *March* 1960)

We have shown (Armin, Grant & Wright, 1960) that the intravascular injection of single small doses of guinea-pig anti-insulin serum produces transient hyperglycaemia in the rat, rabbit and cat. This is thought to be due to the neutralization of endogenously secreted insulin by antibodies in the injected serum. We now show that when larger doses are administered to the conscious rat by intraperitoneal injection or prolonged intravenous infusion, anti-insulin serum produces a diabetic syndrome characterized amongst other things by hyperglycaemia, polyuria, glycosuria and ketonuria.

The rat was chosen for these observations, rather than a larger and experimentally more suitable animal, because of the smaller volumes of serum required; potent anti-insulin serum was in short supply. Moreover, the rat is omnivorous and has already been much used for the study of experimental diabetes produced by alloxan and by pancreatectomy.

METHODS

Details of the methods used for the production and assay of anti-insulin serum have already been reported (Armin *et al.* 1960). Briefly, such serum was obtained from guinea-pigs treated with an emulsion containing recrystallized bovine insulin and its potency was deduced from the hyperglycaemic effect which it evoked in rats on intravenous injection. Control serum was obtained from guinea-pigs treated similarly with insulin-free emulsion, and normal serum was derived from normal untreated guinea-pigs. The rats into which these sera were injected or infused in the present experiments were of an albino Wistar strain and were fed on a standard rat cake diet; unless otherwise stated, food and water were freely available.

Anti-insulin serum. Four batches were used, their potencies being estimated to be such that 1 ml. neutralized approximately 2·3 u. (batch 16204); 3·4 u. (batch 14108); 2·1 u. (batch 227328); and 1·8 u. insulin (batch 424). Control (batches 40 and D15) and normal sera were also used.

Intraperitoneal injection. The rats (male, 190–205 g) were kept in warm cages. Serum (1–5 ml.) was injected through a fine short-bevelled needle into the peritoneal cavity at a point a little to the right of the mid line and half way between the xiphisternum and the

symphysis pubis. Piercing the abdominal wall at this point seldom resulted in injury to, or loss of serum into, the gut. At regular intervals (1–2 hr) after each injection the rats were removed from the cage and placed on a clean glass plate; here they usually passed urine, especially if the end of the tail was compressed. Samples of blood (0·2 ml.) were collected from the cut end of the tail at the same time. At the end of the experiment the rats were reweighed, decapitated and examined.

Intravenous infusion. With the apparatus and method described in the appendix, polythene catheters were inserted into the jugular vein and carotid artery. The rats (male, 230–305 g) were anaesthetized with pentobarbitone sodium.

As soon as the catheters were in place saline infusion was begun, the total rate of intravascular infusion being 0·5 ml./hr. The saline solution consisted of 0·9 % (w/v) sodium chloride in glass-distilled water containing heparin (1000 i.u./100 ml.; Injection of Heparin, Boots Pure Drug Co. Ltd.) and an antibiotic mixture (penicillin, 1000 u. and streptomycin, 100 μg/100 ml.). When placed in the cage the rats recovered consciousness, ate, drank and moved about normally within 1–2 hr. They soon learned to avoid turning round too often in one direction, which caused twisting of the loose skin of the neck around the lower end of the coiled spring.

On the following day, 21–26 hr after insertion of the catheters, guinea-pig serum containing an antibiotic mixture (penicillin, 1000 u. and streptomycin, 100 μg/15 ml.) was infused intravenously for 20 hr. The total fluid infusion rate was increased to 0·82–0·98 ml./hr by adjusting the intra-arterial rate of saline infusion.

When the serum infusion ended, fluid infusion was continued intravenously and intra-arterially at varying rates until the end of the experiment. In most cases saline was infused at total rates ranging from 0·4 to 0·9 ml./hr. In a few experiments (mentioned in detail later) Krebs–Ringer solutions (Umbreit, Burris & Stauffer, 1945) were used to provide potassium or buffering action. At the end of the experiments the rats were killed with an overdose of barbiturate, reweighed and examined.

Investigations

Blood samples (0·2 ml.) were obtained from the tails of injected animals or withdrawn through the arterial catheter of infused rats after discarding the first drops, which were admixed with saline. To avoid excessive blood loss, samples were drawn only when significant changes in blood-sugar concentration were expected. The sugar content was estimated by a modification (Wright, 1957) of the method described by King (1951); the glucose oxidase method (Huggett & Nixon, 1957) was used for some samples and gave essentially the same results.

Urine. All urine passed by infused rats was collected and examined qualitatively; a few quantitative investigations were carried out. Samples passed by injected rats and drops issuing from the funnels under the cages of infused rats immediately after they had micturated were examined qualitatively. Glucose was considered to be present when the urine turned glucose-oxidase test papers ('Clinistix'; Ames and Co.) a deep blue within 30 sec, and ketone bodies if it turned 'Acetest' tablets (Ames and Co.) to a deep violet colour within 10 sec. Slower or less intense colour reactions were considered negative in both tests. To avoid contamination of urine specimens by those passed previously by infused rats, the funnels under the cages were washed and replaced at regular intervals; this was especially important at times when an abnormal component of the urine was expected to disappear.

The urine passed by infused rats was collected in batches over individual periods ranging from 2 to 30 hr and was preserved by the addition of 1 drop of 10 N-HCl at the beginning of each collection period. The volume of each batch was measured and the sugar and ketone-body contents were determined qualitatively by the standard Benedict, Rothera & Gerhardt tests (King, 1951). In some instances the absolute sugar contents were determined

with Benedict's quantitative reagent (King, 1951) and the glucose oxidase method (Huggett & Nixon, 1957); both gave essentially the same results.

Water intake. In all but the first few serum infusion experiments the water bottles fitted to the cages were weighed and refilled at regular intervals. The water consumed by injected rats was not measured.

Food intake. Weighed amounts (50 g) of solid rat cake were placed in the cages of infused rats at regular intervals, the weight remaining at the end of each period giving an indication of the amount eaten. A small amount of broken cake, however, was lost into the funnel. The food consumed by injected rats was not measured.

RESULTS

Intraperitoneal injection

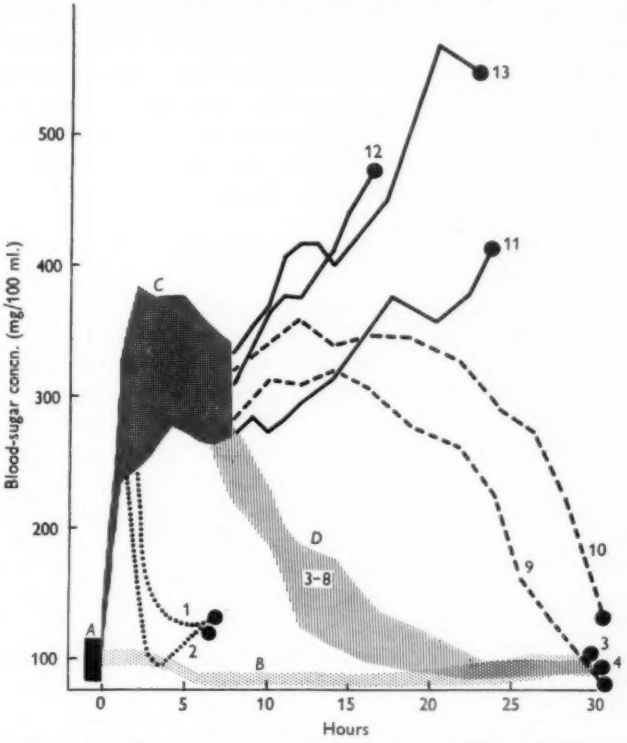
The effects of intraperitoneal injection of guinea-pig serum into groups of conscious rats are shown in Text-fig. 1. Normal and control serum caused no rise in blood-sugar concentration, but marked hyperglycaemia developed within an hour of injecting anti-insulin serum (batch 227328).

Normal and control serum. In two rats injected with 5 ml. normal serum and in two with 5 ml. control serum (batch 40), the blood-sugar concentrations fell slightly but irregularly during the first 8 hr and remained at or a little below the pre-injection level up to 30 hr (Text-fig. 1; area *B*); no observations were made thereafter. During the first 4 hr all rats lost weight (10–15 g) and this was probably due to the unusual amount of faeces passed by them and other injected rats at this stage. Thereafter they maintained weight, eating and drinking water at the same rates as uninjected animals. No abnormality was detected when these rats were killed and examined 55 hr after serum injection.

Anti-insulin serum (1·0 *ml.*). In two rats (Text-fig. 1; rats 1 and 2) injected with this small dose the blood-sugar concentrations rose to maxima (248 and 319 mg/100 ml.) in 1–2 hr but returned almost to the pre-injection levels in $2\frac{1}{2}$–$3\frac{1}{2}$ hr. Transient glycosuria occurred in both rats but neither developed ketonuria. Both rats behaved normally throughout and had lost only 10 g weight when killed 6 hr after injection. No abnormality was detected at necropsy.

Anti-insulin serum (3·0 *ml.*). Four rats (Text-fig. 1; rats 5–8) injected with this medium dose, but otherwise untreated, showed the same initial rapid rise in blood-sugar concentration to maxima ranging from 280 to 370 mg/100 ml. About 5–7 hr after injection the levels began to fall and were fast approaching normal after 10–12 hr. Glycosuria was found in 1–2 hr and ketonuria followed 4–5 hr later. Unlike normoglycaemic rats, they drank frequently and passed urine at about hourly intervals for the first 10 hr. After 10–14 hr, however, all four rats moved about very little and seemed weak; they did not reach up to drink from the water bottles, though they drank when water was brought to their

mouths. They seldom passed urine, and blood samples were obtained with increasing difficulty as blood issuing from the tip of the tail often clotted at once. Despite these signs of dehydration and collapse all four rats recovered. Glycosuria and ketonuria ceased 11–15 hr after injection. After 24 hr two rats from which blood could be obtained were normoglycaemic and all had lost weight (10–20 g); this they regained in the next 3 days, by which time they were behaving normally.



Text-fig. 1. Blood-sugar concentrations in male rats (180–220 g) injected intraperitoneally at zero time with guinea-pig serum. Two rats received control serum (5 ml.) and two were injected with normal serum (5 ml.). The doses of anti-insulin serum injected were 1 ml. (rats 1 and 2), 3 ml. (rats 3–8) and 5 ml. (rats 9–13). Unbuffered Krebs–Ringer solution (1 ml.) was injected subcutaneously after 7 hr every 2 hr into rats, 3, 4, 9 and 10 until they became normoglycaemic again. The shaded areas include all blood-sugar concentrations found (*A*) before serum injection, (*B*) after injection with normal or control serum, (*C*) up to 8 hr after anti-insulin serum injection unless a progressive fall had occurred earlier and (*D*) subsequently in those animals receiving 3 ml. anti-insulin serum. The blood-sugar concentrations at the time of death in animals killed or dying less than 30 hr after injection are indicated by the large dots.

It was thought that the weakness and other symptoms observed in these animals were due to acute dehydration and acidosis. Therefore two more rats (Text-fig. 1; rats 3 and 4) were given the same dose of anti-insulin serum and in addition, starting 7 hr later, were injected subcutaneously every 2 hr with 1 ml. of unbuffered Krebs–Ringer (stock) solution. Essentially the same changes in blood-sugar concentration occurred as in the first four rats, normal levels being re-established in 14–18 hr. Glycosuria started in 1–2 hr with ketonuria following 5–7 hr later; both subsequently ceased 12–14 hr after serum injection. These animals also became weak and stopped eating or drinking, but when glycosuria and ketonuria passed off they began to eat and drink again. Blood samples, however, were easily obtained throughout and the animals urinated every 1–2 hr. When killed 30 hr after injection they still seemed slightly weak and had lost about 20 g weight, but nothing abnormal was found at necropsy.

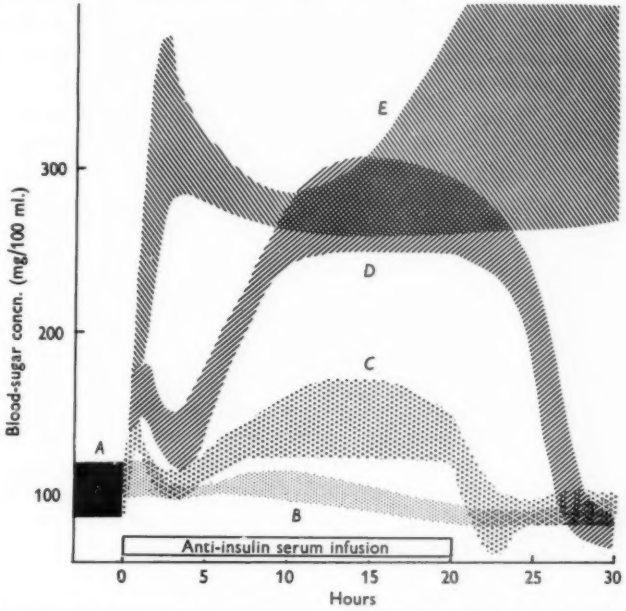
Anti-insulin serum (5 ml.). Of three rats (Text-fig. 1, rats 11–13) injected with this large dose, but otherwise untreated, one died about 17 hr later and two were killed after 24 hr when they seemed about to die. Blood-sugar concentrations rose rapidly, remained between 270 and 370 mg/100 ml. from 2 to 8 hr and then rose to levels greater than 400 mg/100 ml. at death. In the initial stages polyuria and polydipsia occurred; glycosuria appeared within 2 hr with ketonuria 5–7 hr later, both persisting till death. After about 10 hr the animals became weak, but still appeared to be thirsty. Gradually their general condition deteriorated; they became limp, cold to the touch and sunken-eyed. They passed urine seldom and blood was difficult or impossible to obtain. In the 2–3 hr before death their respiratory rates rose to over 140/min. At death they had lost weight (20–30 g); their livers were shrunken and yellow and their stomachs grossly dilated with clear colourless fluid.

Again, two rats (Text-fig. 1; rats 9 and 10) injected with this same large dose (5 ml.) of anti-insulin serum were also given 2-hourly injections (1 ml.) of Krebs–Ringer (stock) solution from 7 hr onwards. The initial response was the same but in neither did the blood-sugar concentration subsequently rise above 360 mg/100 ml. at any time. Marked hyperglycaemia persisted until 16–22 hr, after which time the blood-sugar concentrations fell at first slowly but later rapidly towards normal levels (28–31 hr). Polyuria occurred throughout this hyperglycaemia; glucose appeared in the urine in less than 2 hr, ketone bodies being detected 3–5 hr later. Again the animals became weak but seemed less ill than those which had not received saline injections; their respiratory rates did not rise above 120/min. As the blood-sugar levels fell glycosuria and ketonuria ceased. When the rats were killed (30 hr) they were still

weak and had lost weight (*ca.* 20 g). Necropsy showed that the livers were shrunken and yellow but the stomachs were not distended.

Intravenous infusion

The effects of intravenous infusions of guinea-pig serum into seventeen male rats at various rates for 20 hr are summarized in Text-fig. 2. Normal and control serum had no appreciable effect, whereas anti-insulin serum produced hyperglycaemia of a degree and duration depending upon the rate of serum infusion. When hyperglycaemia persisted for a sufficient time it was accompanied by polyuria, glycosuria, ketonuria and in severe cases led to oliguria, acidosis, marked loss of weight and ultimately to death.



Text-fig. 2. Blood-sugar concentrations in male rats (230–305 g) infused from zero time for 20 hr with guinea-pig serum. The shaded areas include all blood-sugar concentrations found in rats (*A*) before infusion; and up to 30 hr after the start of infusions (*B*) with normal or control serum (0·5–0·6 ml./hr) or with anti-insulin serum at rates of (*C*) 0·14, (*D*) 0·28 and (*E*) 0·5–0·6 ml./hr.

After introduction of the catheters fifteen rats received intravascular saline (0·5 ml./hr) for periods ranging between 21 and 25 hr; two were infused with serum immediately. During this time twelve of these, for which adequate measurements were made, passed urine (mean = 0·50 ml./hr, S.D. = 0·20) and ate food (mean = 0·59 g/hr, S.D. = 0·14) at comparable

rates, but their water intakes (mean = 0·43 ml./hr, S.D. = 0·25) were more variable. Blood-sugar estimations carried out less than 3 hr before infusion with serum showed that all seventeen animals were normoglycaemic (mean blood-sugar concentration = 103 mg/100 ml., S.D. = 8·3), none having levels greater than 120 or less than 89 mg/100 ml.

Control and normal sera (0·5–0·6 *ml./hr*)

Two rats were infused with normal serum and two with control serum (batch D 15) for 20 hr. During this time their blood-sugar concentrations fluctuated irregularly about the pre-infusion level but did not rise above 120 mg/100 ml. (Text-fig. 2; area *B*). The animals moved about, ate, drank and passed urine normally. The urine remained free from glucose and ketone bodies. After infusion with normal serum one animal was infused with saline for 54 hr but was otherwise untreated, whilst the other was starved for 44 hr and infused with saline.

Saline infusion (0·9 *ml./hr*). The blood-sugar concentration never rose above 120 mg/100 ml. The animal continued to pass urine, eat and drink normally; its urine remained free from glucose and ketone bodies. When killed 74 hr after the start of the normal serum infusion the rat had lost some weight (17 g) but necropsy revealed no significant abnormality.

Saline infusion (0·5 *ml./hr*) *and fasting* (44 *hr*). For the first 4 hr this animal was infused with saline alone whilst having free access to food. No significant change in blood-sugar concentration was observed in specimens of blood drawn at regular intervals (cf. changes occurring in animals after infusion with anti-insulin serum at low rate). Thereafter all food, but not water, was withdrawn and the blood-sugar concentration fell steadily over 44 hr to 55 mg/100 ml. Ketone bodies were not detected with 'Acetest' tablets during this period of fasting, but urine collected over the last 24 hr gave a definite pale purple ring in the Rothera test. When food was restored the rat ate hungrily and the blood-sugar concentration rose to 130 mg/100 ml. in less than 2 hr. When killed 79 hr after the start of the serum infusion and 6 hr after the end of the fasting period, some loss of weight (33 g) was noted but necropsy revealed no other abnormality.

Anti-insulin serum

Three groups of rats were infused at different rates with anti-insulin serum, after which they were infused with saline unless otherwise stated.

Low rate (0·14 *ml./hr*). Three rats were infused with serum (batch 227328) at a rate estimated to neutralize approximately 0·29 u. insulin/hr. Text-figure 2 (area *C*) shows that in the first hour the blood-sugar concentration rose by 20–30 mg/100 ml., after which it fell to pre-infusion

levels. At 3 hr a second increase began, the blood-sugar concentration rising 20–70 mg/100 ml. before the serum infusion was stopped. During the serum infusion the rats ate less than usual (0·18–0·30 g/hr) but passed urine and drank at essentially normal rates; they received saline intravenously at a rate of 0·76 ml./hr. None developed glycosuria or ketonuria.

When serum infusion was stopped the blood-sugar concentrations (120–150 mg/100 ml.) fell rapidly by 50–60 mg/100 ml. in 3–4 hr; in each case the pre-infusion level was reached or passed in 3 hr (Text-fig. 2; area *C*). The animals continued to receive intravascular saline (0·51 ml./hr) and though they ate rather less than usual (0·14–0·57 g/hr) they otherwise behaved normally until they were killed 48 hr after the start of serum infusion. By this time they had lost some weight (20–35 g) but nothing abnormal was found at necropsy.

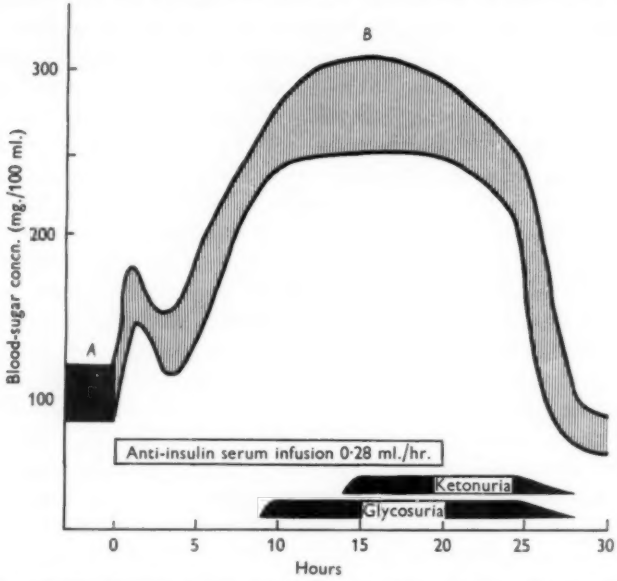
Medium rate (0·28 *ml./hr*). Three rats were infused with serum (batch 16204) at a rate estimated to neutralize approximately 0·64 u. insulin/hr. Text-figures 2 (area *D*) and 3 show that the blood-sugar concentration rose in the first hour to 150–180 mg/100 ml. and then fell. At 4 hr, however, the blood-sugar level began to rise steeply again, reaching 250–280 mg/100 ml. at 10 hr; it remained within the range of 260–310 mg/100 ml. until the infusion was stopped. During infusion with serum the rats ate less (0–0·4 g/hr) and passed more urine (0·59–0·92 ml./hr) but still drank at very variable rates (0·05–0·53 ml./hr); they received saline intravascularly at a rate of 0·62 ml./hr. In each case glucose was first detected in the urine after 9 hr when the blood-sugar concentrations had risen to 230–260 mg/100 ml.; ketone bodies appeared 6 hr later.

When serum infusion was stopped (Text-fig. 3) the blood-sugar concentration remained above 220–250 mg/100 ml. for 3–4 hr before falling sharply to the pre-infusion levels, which were reached at 25–28 hr. Glycosuria and ketonuria ceased almost simultaneously as the blood-sugar concentrations fell below 220 mg/100 ml. Until they were killed 26–33 hr after the end of the serum infusion the rats received intravascular saline (0·6–0·9 ml./hr); they ate (0·66–0·75 g/hr) and drank (0·68–1·24 ml./hr) slightly more than usual and passed variable amounts of urine (0·32–0·92 ml./hr). When killed they had all lost weight (20–40 g) but at necropsy the livers appeared normal and no depletion of body fat was noted.

High rate (0·5–0·6 *ml./hr*). Serum of batches 14108 (four rats), 16204 (one rat) and 424 (two rats) was infused at rates calculated to neutralize approximately 1·1–1·7 u. insulin/hr. During the infusions all seven rats responded in similar fashion, but thereafter their fates varied; six either died or were killed and only one recovered.

Text-figures 2 (area *E*) and 4 show that a rapid uninterrupted increase in blood-sugar concentration occurred in the first 2 hr of the infusion. By

this time levels of 260–380 mg/100 ml. were reached, but subsequently the concentration fell until at 10–12 hr it lay between 260 and 290 mg/100 ml. In five rats the blood-sugar concentration remained between 260 and 310 mg/100 ml. until the end of the infusion but in two others (Text-fig. 4; rats *D* and *F*) higher levels were reached (360 and 390 mg/100 ml.). All the animals moved normally about the cage during the infusion but none of them ate. They passed much urine (0·8–1·9 ml./hr)



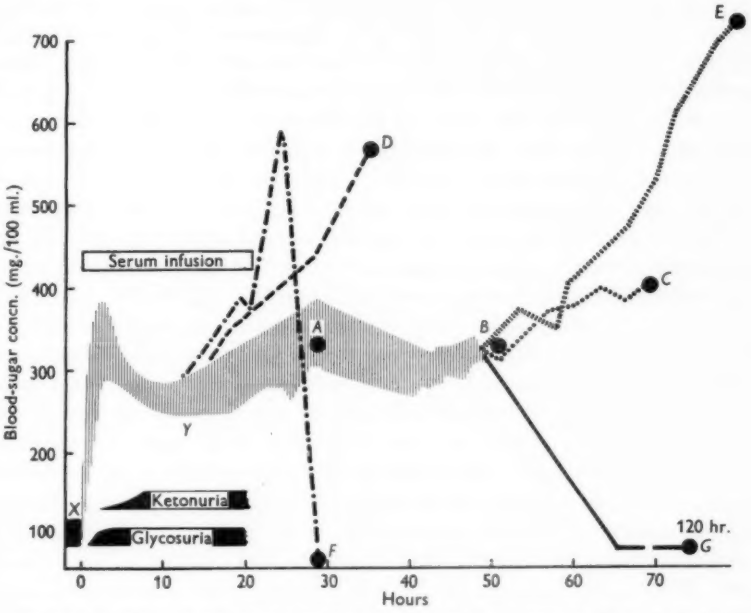
Text-fig. 3. Blood-sugar concentrations, glycosuria and ketonuria in three male rats infused from zero time for 20 hr with anti-insulin serum (0·28 ml./hr). The shaded areas include all blood-sugar concentrations found (*A*) before and (*B*) up to 30 hr after the start of infusions.

and at least one animal drank large volumes of water (1·33 ml./hr). Glucose appeared in the urine within 3 hr and during the infusion its concentration in urine obtained from three rats was about 5 % (Benedict's method). Ketone bodies appeared 3–4 hr after the onset of glycosuria. These animals also received saline (0·3–0·4 ml./hr) intra-arterially.

Some time after the infusions were stopped all but one of the animals died or were killed. The animal which survived (Text-fig. 4, rat G) was the first to be infused and was not observed as closely as the others. The blood-sugar concentration of this animal remained between 260 and 330 mg/100 ml. for a total of at least 50 hr. During this time it passed urine at a high rate (0·97 ml./hr) containing ketone bodies and glucose, the

latter in a concentration of 5–7 %. By 50 hr the animal looked ill and had obviously lost much weight; it was breathing rapidly and its fur was staring. At 65 hr the blood-sugar concentration was normal (80 mg/100 ml.) but the animal still moved slowly and seemed weak. In the next 24 hr it ate and drank well and when killed 5 days after the serum infusion began it was moving actively about its cage. It was not weighed but necropsy showed depletion of retroperitoneal and other fat depots. The liver was mottled and the tissues generally pale and wasted.

One animal (Text-fig. 4; rat *B*) was killed 30 hr after the end of the infusion. This rat was infused with saline at a low rate (0·38 ml./hr) but



Text-fig. 4. Blood-sugar concentrations, glycosuria and ketonuria in seven male rats (*A–G*) infused from zero time for 20 hr with anti-insulin serum (0·5–0·6 ml./hr). The shaded areas include the blood-sugar concentrations found in all rats (*X*) before infusion; and at any one time in the majority of rats (*Y*) during and up to 50 hr after the start of serum infusion. Where the blood-sugar level in a rat differed at any time from those of the majority it is shown separately. The blood-sugar concentrations in individual rats at the time of death are indicated by the large dots. Rats *D* and *F* received Krebs–Ringer solutions intravascularly after infusion with serum and rat *F* also received 12·5 u. insulin I.V. at 24 hr. Ketonuria and glycosuria persisted until normoglycaemia was re-established (rat *G*) or until death; rat *F* was anuric at the time of death and the last urine which it passed at 23 hr contained glucose and ketone bodies.

continued to pass large volumes of urine (1·5 ml./hr) containing glucose in high concentrations (5–7 %) and ketone bodies. Food was still refused and the rat became weak; it was sunken-eyed and limp but continued to respond to stimuli. The blood-sugar concentration remained at 260–340 mg/100 ml. and towards the end of this period some increase in respiratory rate (120–130/min) was noted. When killed 50 hr after the start of the serum infusion the rat had lost much weight (80 g), its liver was small and yellow and the fat depots were depleted. The stomach was markedly distended.

Two other rats (Text-fig. 4; rats *C* and *E*) survived for more than 50 hr and up to that time they behaved similarly. One (rat *E*) was infused slowly with saline (0·12 ml./hr) but also continued to pass urine at a high rate (0·6–0·8 ml./hr) until, at about 58 hr, the rate of urine formation fell rapidly. Up to this point this animal had shown only signs of weakness and dehydration, but then it became progressively more sluggish in its movements. In the last 20 hr of life the blood-sugar concentration rose steadily to more than 700 mg/100 ml. For the last 10 hr no urine was passed. The respiratory rate rose to 130/min and fell again just before the animal was killed. At the time of death, 79 hr after the start of the serum infusion, this rat was thought to be comatose, and was the only one which became so; it lay quietly on its side breathing rapidly and would not respond to stimuli. Apparently about to die, this rat was killed and necropsy revealed the usual findings, a small pale liver, depleted fat depots, a dilated stomach and marked loss of weight (66 g). The other animal (Text-fig. 4; rat *C*) was infused with saline at a greater rate (0·9 ml./hr) and for 30 hr after the end of the serum infusion it too maintained a high urine output (1·4–0·9 ml./hr) and a steady blood-sugar concentration (310–390 mg/100 ml.). Thereafter, however, it passed less urine and became anuric 8 hr before death, which followed a sudden and severe bleeding from the mouth. In these last hours the blood-sugar level rose steadily to more than 380 mg/100 ml., the respiration rate increased (140–150/min) and movements became very sluggish. Necropsy again showed a pale shrunken yellow liver, depleted fat depots and a distended stomach containing blood. There was only slight loss of weight (20 g) but the pleural and peritoneal cavities contained free fluid and the lungs and other tissues generally were grossly oedematous.

One other animal (Text-fig. 4; rat *A*) received only saline (1·1 ml./hr) when serum infusion was stopped. This animal died after two convulsions 29 hr after the start of the serum infusion; necropsy revealed massive haemorrhages into the pericardial and pleural cavities. The rat had lost weight (40 g), its liver had a blotchy appearance and the stomach was slightly distended. Before death the blood-sugar concentration had not

risen above 320 mg/100 ml. and it had been passing urine at a high rate (0·8–1·2 ml./hr).

Since the animals which died in an anuric state showed evidence of potassium depletion and acidosis, attempts were made to prevent these in two other rats. One rat (Text-fig. 4; rat *D*) was infused with unbuffered Krebs–Ringer (stock) solution after the end of the serum infusion when it was seen that the blood-sugar concentration was rising. The rate of infusion (1·5 ml./hr) was calculated to replace fluid lost during the previous infusions of serum and saline. Despite this, however, the blood-sugar concentration continued to rise, anuria developed and the respiratory rate rose until the animal died about 35 hr after the start of the serum infusion. At necropsy the liver had a typical nutmeg appearance and the stomach was slightly distended; the rat had lost weight (43 g). The remaining animal (Text-fig. 4; rat *F*) also had a rapidly increasing blood-sugar concentration at the end of the infusion period. It was, therefore, infused with Krebs–Ringer bicarbonate buffer (1·2 ml./hr). The rat became anuric, its blood-sugar concentration continued to rise, and the blood pressure fell rapidly. Since it seemed certain that this animal would die, it was given insulin (12·5 u.) intravenously, sufficient to counteract half of the anti-insulin serum infused. Within 4 hr the blood-sugar concentration reached hypoglycaemic levels (28 mg/100 ml.) and the blood pressure, already low (45 mm Hg), fell even lower. Six hours after the insulin and 30 hr after the serum infusion began, the rat died in a hypoglycaemic, anuric and hypotensive state, having lost weight (42 g). Necropsy revealed a grossly distended stomach filled with clear colourless fluid, a blotchy congested liver and sparse fat depots.

DISCUSSION

Whether infused intravenously or injected intraperitoneally with anti-insulin serum, all rats whose blood-sugar concentrations rose above about 200 mg/100 ml. developed glycosuria which persisted as long as the hyperglycaemia remained of this degree; the glucose content of such urine ranged between 4 and 7 % (four rats). About 4 hr after the onset of glycosuria ketonuria occurred in every animal whose hyperglycaemia lasted long enough, and passed off again about as soon as did the glycosuria. In the infusion experiments the onset of glycosuria was accompanied by polyuria and probably also by polydipsia. In every case in which serum was given in large amounts the rats stopped eating, but this of itself was not responsible for the ketonuria; even 44 hr fasting provoked but slight ketonuria in an otherwise normal rat.

This syndrome of polyuria, glycosuria, ketonuria (and probably polydipsia) associated with hyperglycaemia differs in at least two respects from

experimental diabetes induced by alloxan in the rat, but is very like that provoked by pancreatectomy. In the first place, permanent hyperglycaemia is not established in the alloxan-treated rat until several hours have elapsed; during this time the blood sugar first rises and then falls, often to hypoglycaemic levels (see Lukens, 1948). Secondly, the persistent hyperglycaemia found in the alloxan-diabetic rat, though accompanied by polyuria, polydipsia and intense glycosuria, is not usually associated with ketonuria, which has been reported by only a few authors (Dunn & McLetchie, 1943; Lyman-Duff & Starr, 1944; Bailey, Bailey & Leech, 1944) or with loss of appetite. Ketonuria also appears to be the exception rather than the rule in alloxan-diabetic rabbits (Lewis, Moses & Schneider, 1947; Duffy, 1945) and dogs (Goldner & Gomori, 1943; Thorogood & Zimmerman, 1945). To study ketonuria, Janes & Myers (1946) chose alloxan-diabetic rats since, as they say, they are usually on the borderline of ketosis and respond more readily to any treatment that alters carbohydrate metabolism than do normal animals. Thus ketonuria and acidosis have been induced in alloxan-diabetic rats by fasting (Guest, Mackler & Knowles, 1952) and by administration of nicotinic acid (Janes & Myers, 1946). Alloxan-diabetic dogs, which also survive for long periods with little evidence of ketonuria (Goldner & Gomori, 1943), rapidly develop ketonuria and fatal acidosis if the pancreas is then removed (Thorogood & Zimmerman, 1945). This latter rapid sequence of events also occurs in the rat after removal of 99·5% of the pancreas (Scow, 1957). Death occurs within 48 hr unless insulin therapy is administered and at the time of death the 'totally' pancreatectomized rat exhibits weakness and prostration, over-ventilation and hypothermia; the stomach also becomes grossly dilated. This is also the picture seen just before death in rats treated with anti-insulin serum and at necropsy the stomach is also grossly distended.

After pancreatectomy or treatment with alloxan the persistent diabetic state is due to more or less complete destruction or removal of the β-cells which secrete insulin. It seems, though the evidence at present is only circumstantial, that the diabetic state induced by anti-insulin serum is not due to any failure of insulin production by the pancreas but rather to neutralization of all endogenously secreted insulin by the injected antibody. It is known that injected labelled antibodies persist in decreasing amounts in the circulation (Coons, 1954) and so presumably an animal injected with anti-insulin serum will remain diabetic until its pancreas has secreted sufficient insulin to neutralize the antibody. In the mean time secondary metabolic changes develop which may cause death. Dehydration and weakness, which prevented voluntary drinking in the intraperitoneally injected rats, resulted in acidosis and death when the dose exceeded 3 ml. Life was prolonged and recovery facilitated by administration of fluid

subcutaneously. Saline infusion prolonged the lives of rats infused with larger doses of anti-insulin serum to 70–80 hr and one rat recovered. All those which died or were killed after prolonged hyperglycaemia became oliguric and then anuric by which time they showed signs of acidosis. Diabetic acidosis is associated with potassium depletion; therefore two rats (Text-fig. 4; rats *D* and *F*) were infused with solutions containing bicarbonate buffer or potassium as soon as the blood-sugar concentration was found to be rising rapidly. This treatment, however, did not seem to prolong life or to restore renal function. It may be that these particular animals, which were used in midwinter, were not so physically fit as those which were infused in the late summer (Text-fig. 4; rats *B*, *C*, *E* and *G*) and which developed oliguria at a much later stage. All that can be said at the moment is that it is possible to maintain diabetic rats alive for about 30 hr and that on occasion they have been kept alive for almost 80 hr. The factors which facilitate recovery are not yet known, but at least one of the animals used in the present experiments did recover after being diabetic for at least 50 hr whilst receiving only intravenous saline.

The responses to different doses of anti-insulin serum suggest that the islet cells of the pancreas react in two phases; first, they release stored insulin and then they secrete the hormone as fast as it is required or can be synthesized. Such a store of hormone has been inferred from the presence of granules which disappear from the β-cells when glucose is administered (see Lazarow, 1957). It could also be inferred from the fact that the effects produced by anti-insulin serum were not directly proportional to the dose injected. Thus 5 ml. injected intraperitoneally provoked hyperglycaemia lasting approximately 30 hr whilst that following 1 ml. lasted only about 3 hr (Text-fig. 1). Further, infusions at the lower rates (0·14 and 0·28 ml./hr) caused an initial rise in blood-sugar concentration which was followed by a temporary fall and then a greater and more persistent hyperglycaemia (Text-fig. 2). We interpret these observations to mean that the initial hyperglycaemia induced by the injection or infusion of the anti-insulin serum causes the release of insulin stored in the β-cells; this neutralizes small injected doses and halts the progressive increase in blood-sugar concentration induced by serum infused at low rates. Once this store of insulin has been released and neutralized, the blood-sugar concentration again rises in the infused animals if endogenously secreted insulin is insufficient to neutralize the infused antibody and supply the requirements of the tissues.

Assuming that this interpretation is true, that the estimates of insulin-neutralizing power of the serum are correct and that all the antibody in the injected serum remains available to neutralize insulin, we may calculate the rate at which the rat pancreas can secrete the hormone. Rats weighing

approximately 250 g when infused with serum at a rate sufficient to neutralize about 0·3 u. insulin/hr, developed slight but persistent hyperglycaemia towards the end of the infusion period, after which the blood-sugar concentration fell immediately (Text-fig. 2; area *C*). This implies that they were producing sufficient insulin to neutralize the infused antibody but not quite enough to maintain normoglycaemia as well. Higher rates of infusion sufficient to neutralize about 0·64 u. insulin/hr produced a more marked secondary hyperglycaemia, which persisted for several hours after the end of the infusion (Text-fig. 2; area *D*). In this case the pancreas was unable to secrete enough insulin even to neutralize the infused antibody. It seems, then, that the rat pancreas can secrete insulin at a rate between 1·2 and 2·6 u./kg/hr. The effects produced by intraperitoneal injection of the serum support this conclusion. Hyperglycaemia lasting about 15 hr was induced by 3 ml. serum, whilst 5 ml. elevated the blood-sugar level for 30 hr (Text-fig. 1). The additional 2 ml. of serum, sufficient to neutralize about 4·2 u. insulin, prolonged hyperglycaemia for 15 hr in rats weighing 200 g. Thus towards the end of the hyperglycaemic period these rats were secreting insulin at a rate of about 1·4 u./kg/hr. These rough estimates of the probable maximum rate at which the pancreas is capable of synthesizing and secreting insulin are much greater than the 0·33–0·50 u./kg/hr of soluble insulin which Spiro & Hastings (1958) found necessary to maintain normal metabolism in alloxan-diabetic rats once adequate control of the diabetes had been established. If this latter rate of insulin dosage represents its normal insulin requirements, then the rat can apparently increase its insulin secretion rate three- to fivefold and can maintain this rate for many hours. It remains to be seen, however, whether more prolonged stimulation of the pancreas by hyperglycaemia will exhaust and finally destroy the secretory capacity of the β-cells.

Similarly the reserve of insulin in the pancreas can be calculated roughly. When anti-insulin serum is infused at a rate sufficient to neutralize 0·64 u. insulin/hr, the initial rise in blood sugar concentration is halted after 1 hr for 2–3 hr before the secondary rise begins (Text-fig. 2; area *D*). Presumably, therefore, the pancreas has released sufficient insulin to neutralize the antibody infused during this period. On this basis the reserve of insulin available in such rats (250 g) is about 5–8 u./kg. Further, the injection of serum sufficient to neutralize 2·1 u. insulin produced hyperglycaemia in 200 g rats lasting only 3 hr (Text-fig. 1; rats 1 and 2); insulin must therefore have been secreted at a rate of 3·5 u./kg/hr. Thus even if the pancreas had been synthesizing and secreting insulin at its maximum rate (1·4 u./kg/hr) it must also have secreted an additional 6·3 u./kg during this time, and this may be taken to represent a low estimate of the insulin reserve in the pancreas. For comparison with these

rough estimates, it may be noted that the mean extractable insulin in the pancreas of the Wistar rat is 7·5 u./kg and ranges between 5·8 and 8·4 u./kg (Best, Haist & Rideout, 1939).

These preliminary observations suffice to show that guinea-pig anti-insulin serum provides a useful tool for producing a diabetic state and for studying insulin metabolism. More extensive experiments with animals larger than the rat would be possible if potent anti-insulin serum were available in greater amounts. At present the guinea-pig is the only animal known to yield active serum. It may be possible both to increase the titre by modifying existing production methods and also to increase the yield of serum by finding a bigger animal with an immunological response to insulin similar to that of the guinea-pig.

SUMMARY

1. Anti-insulin serum obtained from guinea-pigs treated with bovine insulin has been injected intraperitoneally and infused intravenously for 20 hr into conscious rats.

2. Provided the dose is sufficient, such serum induces a diabetic syndrome characterized by hyperglycaemia, glycosuria, polyuria, ketonuria and loss of appetite, and leading in some cases to loss of body weight, oliguria, anuria and death and in others to recovery. This syndrome is similar to that found in the 'totally' pancreatectomized rat but differs from alloxan diabetes.

3. The results suggest that the rat is capable of producing endogenous insulin at a rate of approximately 1·2–2·6 u./kg/hr and maintains a store of insulin in the pancreas (5–8 u./kg) which it can secrete rapidly.

We wish to thank Dr M. A. Floyer of the London Hospital whose infusion pump and apparatus, exhibited at a meeting of the Medical Research Society in October 1958, formed the basis of ideas incorporated in the apparatus used in the present experiments. We also wish to thank the Medical Research Council for a grant and Miss Sheila Haizelden, Miss Ann Buxton and Mr Kenneth Kilbourn for technical assistance.

REFERENCES

Armin, J., Grant, R. T. & Wright, P. H. (1960). Acute insulin deficiency provoked by single injections of anti-insulin serum. *J. Physiol.* **153**, 131–145.

Bailey, C. C., Bailey, O. T. & Leech, A. B. (1944). Alloxan diabetes with diabetic complications. *New Engl. J. Med.* **230**, 533–536.

Best, C. H., Haist, R. E. & Rideout, J. H. (1939). Diet and the insulin content of the pancreas. *J. Physiol.* **97**, 107–119.

Coons, A. H. (1954). Labelled antigens and antibodies. *Annu. Rev. Microbiol.* **8**, 333–352.

Duffy, E. (1945). Alloxan diabetes in the rabbit. *J. Path. Bact.* **57**, 199–212.

Dunn, J. S. & McLetchie, N. G. B. (1943). Experimental alloxan diabetes in the rat. *Lancet*, **245**, 384–387.

Goldner, M. G. & Gomori, G. (1943). Alloxan diabetes in the dog. *Endocrinology*, **33**, 297–308.

GUEST, G. M., MACKLER, B. & KNOWLES, H. C. (1952). Effects of acidosis on insulin action and on carbohydrate and mineral metabolism. *Diabetes*, **1**, 276–282.

HUGGETT, A. ST G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase and *o*-dianisidine in determination of blood and urinary glucose. *Lancet*, **273**, 368–370.

JANES, R. G. & MYERS, L. (1946). Production of ketosis in alloxan diabetic rats with nicotinic acid. *Proc. Soc. exp. Biol., N.Y.*, **63**, 410–414.

KING, E. J. (1951). *Micro-analysis in Medical Biochemistry*, 2nd ed. London: Churchill.

LAZAROW, A. (1957). Cell types of the Islets of Langerhans and the hormones they produce. *Diabetes*, **6**, 222–232.

LEWIS, L. A., MOSES, J. & SCHNEIDER, R. W. (1947). Alternation in alloxan diabetic rabbits especially in relation to ocular damage. *Amer. J. med. Sci.* **213**, 214–220.

LUKENS, F. D. W. (1948). Alloxan diabetes. *Physiol. Rev.* **28**, 304–330.

LYMAN-DUFF, G. & STARR, H. (1944). Experimental alloxan diabetes in hooded rats. *Proc. Soc. exp. Biol., N.Y.*, **57**, 280–282.

SCOW, R. O. (1957). 'Total' pancreatectomy in the rat; operation, effects and post-operative care. *Endocrinology*, **60**, 359–367.

SPIRO, R. G. & HASTINGS, A. B. (1958). Studies of carbohydrate metabolism in rat liver slices. XI. Effect of prolonged insulin administration to the alloxan-diabetic animal. *J. biol. Chem.* **230**, 751–759.

THOROGOOD, E. & ZIMMERMANN, B. (1945). The effects of pancreatectomy on glycosuria and ketosis in dogs made diabetic by alloxan. *Endocrinology*, **37**, 191–200.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*. Minneapolis: Burgess.

WRIGHT, P. H. (1957). Plasma insulin estimation by the rat diaphragm method. *Lancet*, **273**, 621–624.

APPENDIX

APPARATUS AND METHOD FOR PROLONGED INTRAVASCULAR INFUSION OF CONSCIOUS RATS

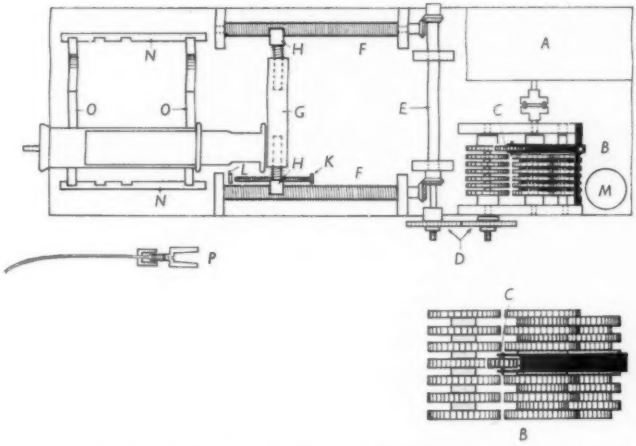
BY J. ARMIN

In each experiment two rats (230–305 g) are anaesthetized (pentobarbitone sodium, 30 mg/kg; 0·5 ml./kg Veterinary Nembutal, Abbott Laboratories Ltd.) and in each catheters are inserted in an external jugular vein and a carotid artery. They are then transferred to cages (Plate 1) and infused with saline or guinea-pig serum delivered from an infusion pump (Text-fig. 5).

Catheterization. For each rat two catheters are prepared, each consisting of polythene tubing (internal diameter, 0·011 mm; size P.E.10; Clay Adams Co. Inc., New York), 35–40 cm long, with one end bevelled and the other flared for connexion by means of a screw cap to one nozzle on a three-way Perspex tap (Armin & Grant, 1953). The second nozzle of this tap is connected by larger polythene tubing (internal diameter, 0·023 mm; size P.E.50) to the syringe of a perfusion pump (see below). To the third nozzle is attached a short length (1 cm) of tubing (size P.E.50) with a flared open end into which a blunt syringe needle may be fitted; this tubing can be connected to a capacitance manometer for blood pressure measurements and through it blood samples may be obtained or fluids

injected. The two catheters are threaded through a closely coiled stainless-steel wire spring (24 S.W.G.; coil internal diameter 3 mm; length 18 cm). The last coil at the end distal to the three-way tap is expanded to a diameter of 10 mm and covered with polythene tubing. The syringe and connected catheters are filled with saline and freed from air.

Through a $\frac{1}{2}$ in. (13 mm) incision at the back of the neck the catheters are passed through a passage in the subcutaneous tissues to a second $\frac{1}{2}$ in. incision at the front of the neck. They are inserted for a distance of 1–3 cm into the carotid artery and external jugular vein previously isolated



Text-fig. 5. Diagram of infusion pump, syringe and polythene tubing connexion; details given in text.

on one side, and each secured in place with two ligatures. The saline infusion is then started and the anterior neck wound closed. The expanded end of the spring is fixed beneath the skin in the dorsal incision by a purse-string suture and the remainder of the incision is closed. The rat is then transferred to the cage.

The cage and attachments (*Plate* 1). A wire cage ($45 \times 30 \times 15$ cm) is divided into two compartments and provided at its sloping front with a common door opening downwards. It rests on a metal stand which also supports at one side the two perfusion pumps. At the centre of the top of each compartment is a hole (3×3 cm^2) above which is a gantry consisting of two vertical brass rods (10 cm $\times$ 0·6 cm) joined at the top by a horizontal Perspex plate (Plate 1). Between, and moving up and down at its slotted ends upon these rods, is a Perspex bar, in the centre of which is a slot whose width can be adjusted with a screw; the upper end of the

coiled spring is fixed in this slot. The centre section of the horizontal bar is free to move about a horizontal axis in the plane of the vertical rods, and is suspended by a cord running over a pulley in the Perspex plate above and attached to a counterweight. Once the three-way taps have been closed and disconnected from the tubes leading to the infusion pump, they can be passed through the hole in the roof when the anaesthetized catheterized rat is transferred to the cage. The upper end of the coiled spring is fixed in the movable horizontal bar. The taps are screwed into position on the horizontal Perspex plate and reconnected to the pump for immediate infusion with saline. This system allows the rat to move about the cage, the coiled spring moving up and down freely without exerting tension or pressure on the rat's neck. The only movement which is restricted is rotation of the coiled spring about its own axis, but the animal soon learns not to turn round too often in one direction.

Immediately beneath each compartment of the cage is fitted a removable celluloid funnel with a rectangular top of the same dimensions as the base of the compartment. Below the funnel spout stands a conical flask (50 ml.) around the mouth of which are three small teeth. Resting on these teeth is a pear-shaped glass ball whose diameter is slightly greater than that of the mouth of the flask and whose pointed end is directed into the flask. Faeces emerging from the funnel strike this ball and fall clear, whereas urine passes round and is collected in the flask beneath. To prevent urine escaping from the cage, a strip of celluloid (5 cm wide) runs round the bottom of each compartment at an angle of 45°. Above this celluloid strip the tube from a water bottle projects into each compartment.

Perfusion pump with variable gears (*Text-fig.* 5). The following apparatus has proved convenient and reliable for the prolonged infusion of small amounts of fluid into small animals. The whole is mounted on a duralumin base plate ($13 \times 36 \times 0{\cdot}5$ cm). *A* is a synchronous motor ('Sectric' heavy-duty clock motor, No. 55697; Smith, London) giving one shaft revolution per minute. The shaft is connected to *B*, a combination gear train giving 7 stages from a direct drive, by 2 to 1 reductions to a final reduction of 64 to 1. The small wheel *C* selects the gear required and transmits the drive to a pair of gear wheels *D*, which can be changed at will from direct drive, to 3 or 5 to 1 reduction or 1 to 3 or 5 increase. *D* drives the shaft *E* which in turn drives the pair of screws *F*, 20 threads to the inch. Between the screws is the ram *G* on each end of which is a spring-loaded shaft ending in a half-nut *H* which fits on to the screws. Mounted on the underside of one of the half-nuts is an adjustable screw *K*, so arranged that when the ram is near the end of its traverse, it impinges on the arm *L* of a switch (Microswitch V3ML; Burgess Products Co. Ltd., Gateshead) mounted underneath the base plate, which breaks the current to the motor and

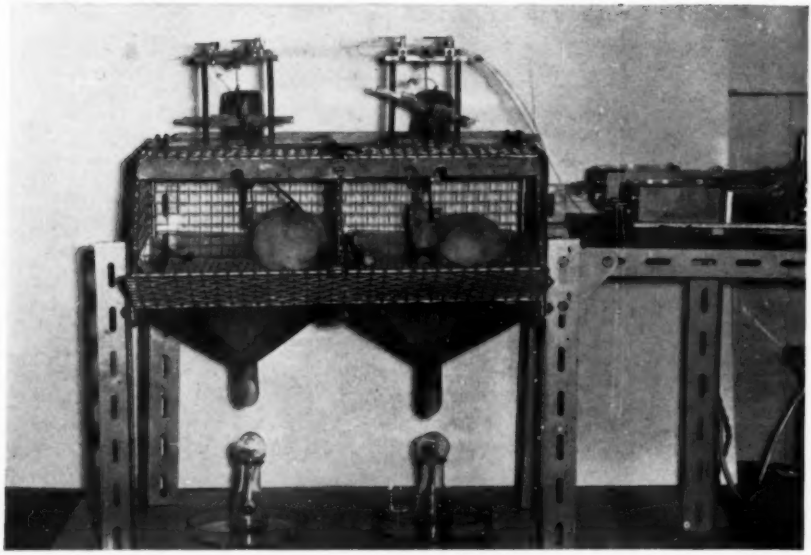


Fig. 1

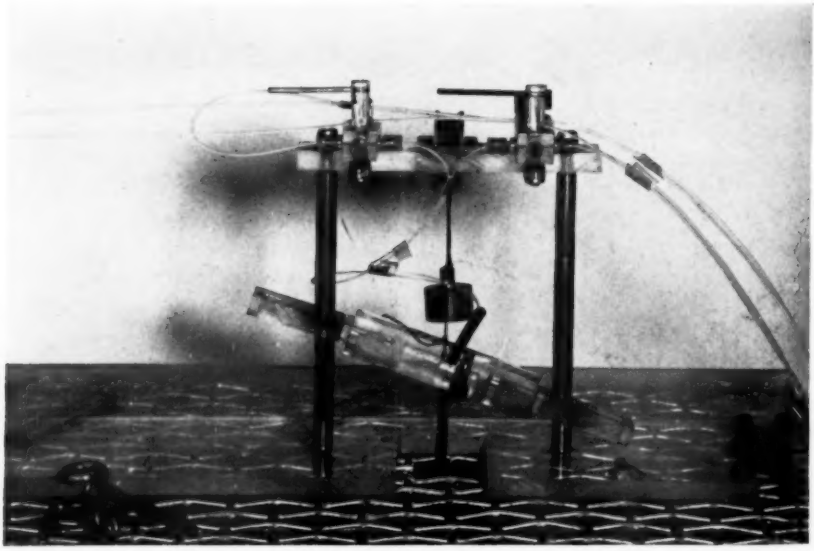


Fig. 2

lights a warning lamp *M* or rings a bell. *N* is a pair of parallel duralumin plates with slots cut in the opposing faces to hold two 0·64 cm Perspex slides *O*. The slides have U-shaped cut-outs into which glass syringes fit snugly. Those now in use can accommodate four 1 ml. syringes, three 2 or 5 ml., two 10 or 20 ml. or one 50 ml. syringe. The Perspex cap joining the syringe nozzle to the polythene tubing used for infusion is shown exploded at *P*. A Perspex cap fits over the syringe nozzle and ends in a screw. The end of the polythene tube is passed through a cap nut and flared with gentle heat. The cap nut is then screwed on to the Perspex cap. The syringe piston must fit snugly in the barrel and should be ground throughout its length. The piston is lubricated with silicone high-vacuum grease (W. Edwards and Co. Ltd., London). These measures prevent loss of fluid between piston and barrel. The gearing leads to a forward drive of the ram of from 381 to 0·238 mm/hour. The volumes delivered by the various syringes can therefore range in 210 steps from about 232 to about 0·004 ml./hr.

REFERENCE

ARNIM, J. & GRANT, R. T. (1953). The artery of the denervated rabbit's ear as a sensitive pharmacological test object. *J. Physiol.* **121**, 593–602.

EXPLANATION OF PLATE

PLATE 1

Fig. 1. Cage for infusion of conscious rats; for details see text.

Fig. 2. Gantry fixed above cage for support of coiled spring carrying catheters and attachment of three-way taps; for details see text.

J. Physiol. (1960), **153**, *pp.* 166–178
With 1 *text-figure*
Printed in Great Britain

THE EFFECTS OF SUBCUTANEOUS FAT AND OF PREVIOUS EXPOSURE TO COLD ON THE BODY TEMPERATURE, PERIPHERAL BLOOD FLOW AND METABOLIC RATE OF MEN IN COLD WATER

By W. R. KEATINGE*

From the Medical Research Council Department of Experimental Medicine, University of Cambridge

(*Received* 1 *April* 1960)

Individual fat men have been observed to lose less heat than thin men in cold surroundings (Winslow, Herrington & Gagge, 1937; Pugh & Edholm, 1955; Carlson, Hsieh, Fullington & Elsner, 1958; Keatinge, 1959). It has been reported that men maintained their peripheral blood flow better when exposed to cold sitting still indoors, after a period of physical training in a cold climate, than they did in a similar experimental exposure to cold before this period of training in a cold climate (Brown & Page, 1952–3; Keatinge & Evans, 1958). In the present experiments men with a wide range of fat thickness have been immersed repeatedly for periods of 30 min in water at 15° C, in order to determine whether their falls in deep body temperature during immersion depended mainly on the thickness of their subcutaneous fat or mainly on their peripheral blood flow, and whether these falls were increased by exposure to cold or by exercise earlier in the day.

Reports about the effect of acclimatization to cold on men's metabolic rates in the cold have been conflicting (Burton, 1941; Adolph & Molnar, 1946; Le Blanc, 1956) but it has been found (Keatinge, 1959) that repeated brief immersion in water at 15° C reduced men's over-ventilation and increase in heart rate, as well as their metabolic rate, during such immersion. Metabolic rates have been measured in the present experiments in the hope of throwing light on the mechanism and significance of such changes. The experiments have also provided information about the method by which the metabolic rates of fat and thin men were adjusted to their differing rates of heat loss in the cold.

* Elmore Student.

METHODS

The subjects were twelve healthy naval ratings, all volunteers, aged between 18 and 28. They came in groups of four and each group was studied over a period of 3 weeks. On the first day their heights and weights were measured, their subcutaneous fat thicknesses were estimated by Harpenden callipers (Edwards, Hammond, Healey, Tanner & Whitehouse, 1955) at the sites used previously (Keatinge, 1959) and they were given practice in wearing the masks used for the immersion experiments.

All immersions were made in an indoor tank, 8 ft. long and 4 ft. (1·22 m) wide and deep, which has already been described (Keatinge, 1959). During the next 3 weeks each man was repeatedly immersed up to his shoulders in this for 30 min at a time. The water was always at $15 \pm 0{\cdot}1°$ C and was vigorously stirred mechanically; while immersed in it the man sat on a slatted wooden seat, wearing only very brief cotton bathing trunks, a helmet and a mask. Under these conditions the temperature of the immersed skin was shown to fall in 2 min to within 1° of 15° C (Keatinge, 1959). Before immersion each man sat unclothed for at least 30 min in the room, which was maintained by a thermostat at $29 \pm 1°$ C in all except the 'initially hot' and 'initially cool' experiments.

The man's rectal temperature was followed by a thermojunction enclosed in semi-rigid plastic tubing and inserted 11 cm from the anus. The cold junction was in a vacuum flask of ice and water and the e.m.f. was measured by a potentiometer; readings were accurate to the nearest 0·05° C. The air expired during the first 10 and last 20 min of the 30 min immersions was collected separately by masks and either passed into a Max Planck Institute Respirometer or into Douglas bags. Its oxygen deficit was then measured by a Hartmann and Braun automatic analyser. The meter was accurate to $\pm 1\%$ and the analyser to $\pm 2\%$. The same meter was used to measure the volume of gas in the Douglas bags when the latter were used. The metabolic rates were then calculated by the method of Weir (1949). These rates, particularly those determined over the first 10 min of immersion, were subject to error, for the men were not in a steady state of oxygen exchange, but this error can only have been small and was presumably consistent for the same individual in different experiments. The oxygen deficit of the expired air was used as an indication of the degree of over-ventilation in relation to oxygen consumption. An electrocardiograph record was obtained from waterproof leads on the wrists for the whole of the first 15–70 sec of immersion, and subsequently for the first 15 sec of every minute. In order to estimate peripheral blood flow during immersion a heat-flow disk (Hatfield, 1949) was stuck by 'Nobecutane' (Evans) to the dorsum of the terminal phalanx of the index finger of the right hand and supported by a rubber band around its base. This finger was immersed in the tank for 10 min before the start of each experiment to permit an estimate of blood flow in the finger to be made just before immersion, when there was no general cooling of the body.

During either their 2nd or their 3rd day each man was immersed twice. Before one of these immersions (initially cool) the room temperature was 24–27° C, and before the other (initially hot) was 38–39° C. The men sat in the room at these temperatures for 30 min before immersion, and at the higher temperature they were always sweating before immersion. Subjects with odd numbers were immersed in the morning with the room hot, and then in the afternoon of the same day with the room cool; those with even numbers were immersed in the opposite order.

During the next 8 days each man was immersed regularly every second day, always in the afternoon between 2.30 and 4 p.m. During the mornings before an immersion, the men either (*a*) sat still, normally clothed, in a warm room at $23 \pm 2°$ C, (*b*) sat still, wearing only shorts, in a room at 5° C, (*c*) did a stepping exercise (18 cm, 24/min) in the warm room or (*d*) in the cold room. They sat in the cold room, or did the exercise, for three periods of 50 min during the morning, with two 10 min breaks in which they rested in the warm. The last of the periods finished at 11.45 a.m. The men were able to hold a fixed bar during the

stepping exercise, and so to use their arms as well as their legs. These four experiments were crossed over in a 'Latin Square' pattern for each group of four men. Two men in the second group, however, were injured in a car accident during the second week, and had to be dropped from the later experiments; the results from the two remaining men are only fully 'crossed-over' as regards comparison of the effect of 'warm' and 'cold' mornings, and results are available on only eight men for comparing the effect of 'working' and 'still' mornings. In most of the tables the means of the results for these eight men are given as well as the means for all the men, in order to facilitate a general comparison of the results from all types of experiment. The order in which each man went through each group of experiments is shown in Table 3. The men took no exercise on the day of an immersion except for that prescribed; they were always driven from the naval unit where they lived to the laboratories, and were kept under observation on the mornings before immersions.

In the statistical analysis all the results were assumed to be normally distributed except values for heat loss from the finger, which frequently included a number of values much higher than the mean and were therefore subjected to non-parametric analysis by the sign test.

TABLE 1. Ages, heights, weights and fat thickness of the subjects

				Skinfold thickness (mm)				
Subject	Age (years)	Height (cm)	Weight (kg)	Sub-scapular	Abdomen	Biceps	Subcostal	Average for subject
				(means of 4 determinations)				
1	18	175	88·4	35·9	31·2	9·3	30·9	26·8
2	23	167	69·4	10·9	8·2	3·8	9·3	8·1
3	23	178	67·1	14·0	12·9	3·7	14·4	11·2
4	22	176	66·2	6·5	6·0	2·8	6·2	5·4
5	19	170	61·7	8·6	6·6	3·4	7·0	6·4
6	18	174	63·7	8·3	8·8	3·8	7·5	7·1
7	19	183	86·6	13·1	13·9	6·1	11·1	11·1
8	19	169	64·4	8·3	8·3	3·4	8·7	7·2
9	22	181	63·9	5·6	4·9	2·5	4·4	4·4
10	22	180	73·5	9·6	7·1	3·8	6·7	6·8
11	19	176	73·0	11·9	10·5	5·3	10·8	9·6
12	19	171	67·1	8·2	6·7	3·7	7·2	6·5
Mean	20·3	175·0	70·42	11·74	10·43	4·30	10·35	9·22

RESULTS

Table 1 gives the ages, heights and weights of the subjects and their skinfold thicknesses; each skinfold thickness reading represents the mean of four readings at each site, two from each side of the body. Subject 1 had by far the greatest mean skinfold thickness, 26·8 mm, and was also the heaviest, while the skinfold thicknesses of the remaining men varied from 4·4 to 11·2 mm.

Table 2 shows that when the men were exposed to an ambient temperature of 38–39° C for half an hour before immersion their rectal temperatures at the time of immersion were on average 0·31° C higher, their skin temperatures 3·5° C higher, their heart rates were higher by 14·7 beats/min and the rates of heat loss from their index fingers in water at 15° C, used as an index of blood flow, were always much higher than when they had previously been in a cool room. These differences were all significant,

often highly so. There were no significant differences in any of these measurements before different experiments in the subsequent four immersions, although the skin and rectal temperatures tended to be slightly higher after 'warm' than 'cold' mornings.

TABLE 2. Rectal temperatures, skin temperatures, heart rates and finger heat loss before immersion. $S\bar{d}$ = sample standard error of difference between two sets of results

			Activity during morning before immersion			
	Initially hot	Initially cool	Still, warm	Still, cold	Working, warm	Working, cold
Rectal temperatures (°C)						
Mean (all subjects)	37·98	37·67	37·80	37·70	37·81	37·77
Range	37·45–38·50	37·15–38·45	37·20–38·20	37·20–38·15	37·40–38·25	37·65–38·00
	Difference $S\bar{d}$ 0·133 $P < 0·05$					
Mean for subjects 1–4 and 9–12	38·00	37·68	37·74	37·71	37·83	37·78
Mean skin temperatures (°C)						
Mean (all)	35·80	32·30	34·58	34·35	34·86	34·73
Range	34·24–36·87	30·10–33·87	33·73–35·41	33·13–35·39	33·93–37·73	33·77–35·82
	Difference highly significant					
Mean for subjects 1–4 and 9–12	—	—	34·76	34·38	35·18	34·92
Heart rates (beats/min)						
Mean (all)	97·4	82·7	82·4	83·6	86·5	85·8
Range	70·5–114·3	55·5–104·2	75·9–93·4	70·8–102·9	71·4–96·8	69·0–106·7
	Difference $S\bar{d}$ 3·40 $P < 0·05$					
Heat loss from index finger (cal/cm²/min)						
Mean (all)	0·318	0·016	0·121	0·051	0·079	0·048
Range	0·070–0·911	0·004–0·042	0·014–0·666	0·009–0·105	0·021–0·158	0·018–0·082
	Difference highly significant					
Mean for subjects 1–4 and 9–12	—	—	0·052	0·045	0·075	0·046

Tables 3 and 4 show that the rectal temperatures fell faster in the water when the men were initially hot than when they were cool. The difference was significant but it was small, and even at the end of the experiment the rectal temperatures were still higher when the men were hot than when they were cool at the time they were immersed. The tables also show that the falls in temperature were not significantly altered by exercise or exposure to cold during the morning before immersion, and that although the temperatures of different men fell at very different rates, the fall in the rectal temperature of each man varied little in these four experiments. Figure 1 shows that the average fall in the rectal temperature of each man during these four immersions was closely dependent on his mean skinfold thickness; there was an approximately linear relationship, which was highly significant ($P < 0·001$), between the men's falls in temperature and the reciprocal of their mean skinfold thickness. The relationship is given by the equation $\Delta T = 11·7/F - 0·62$, where ΔT is the fall in rectal temperature (°C) and F the mean skinfold thickness (mm).

Table 5 shows that 8 min after immersion the rate of heat loss from the

men's index fingers was always very low; it always remained low until the end of the experiment. It was always greater when the men were hot than when they were cool at the time of immersion but was not affected by prior exercise or exposure to cold. Some small differences between the rate at which the men's rectal temperatures fell in the latter experiments were associated with differences in peripheral blood flow. This is best

TABLE 3. Fall in rectal temperature (°C) during 1st 10 min of immersion

			Activity during morning before immersion				
Subject	Initially hot	Initially cool	Still, warm	Still, cold	Working, warm	Working, cold	Mean for individual
1	0·20† (1)*	0·05 (2)	0·05 (3)	0·10 (1)	0·10 (4)	0·10† (2)	0·04
2	0·35 (2)	0·30 (1)	0·05 (4)	0·20 (2)	0·10 (3)	0·15 (1)	0·13
3	0·40 (1)	0·05 (2)	0·05 (1)	0·20† (3)	0·15 (2)	0·10 (4)	0·03
4	0·45 (2)	0·35 (1)	0·20 (2)	0·20 (4)	0·25 (1)	0·30 (3)	0·24
5	0·05† (1)	0·25 (2)	0·10 (3)	0·05† (1)	0·05 (4)	0·35 (2)	0·11
6	0·30 (2)	0·20 (1)	—	—	—	—	—
7	0·05† (1)	0·00 (2)	0·10 (1)	0·00 (3)	0·05 (2)	0·05 (4)	0·05
8	0·15 (2)	0·10 (1)	—	—	—	—	—
9	0·60 (1)	0·40 (2)	0·05 (3)	0·20 (1)	0·30 (4)	0·20 (2)	0·19
10	0·40 (2)	0·10 (1)	0·10 (4)	0·10 (2)	0·10 (3)	0·00 (1)	0·08
11	0·05† (1)	0·00 (2)	0·00 (1)	0·00 (3)	0·15† (2)	0·00 (4)	0·04†
12	0·45 (2)	0·25 (1)	0·10 (2)	0·10 (4)	0·15 (1)	0·00 (3)	0·09
Mean (all subjects)	0·23	0·17	0·08	0·06	0·11	0·11	
Mean for subjects 1–4 and 9–12	0·30	0·19	0·08	0·09	0·13	0·08	

* Numbers in brackets indicate order in which experiments were made on each man in each series. † After a number denotes a rise in temperature.

TABLE 4. Fall in rectal temperature (°C) between 10th and 30th minute of immersion

			Activity during morning before immersion				
Subject	Initially hot	Initially cool	Still, warm	Still, cold	Working, warm	Working, cold	Mean for individual
1	0·15	0·10	0·10	0·10	0·15	0·10	0·11
2	0·10	0·85	0·95	0·80	1·00	0·85	0·90
3	0·55	0·55	0·45	0·25	0·10	0·40	0·30
4	1·35	1·20	1·40	1·50	1·80	1·45	1·54
5	0·85	0·80	1·00	0·85	1·00	0·80	0·91
6	0·80	0·85	—	—	—	—	—
7	0·55	0·15	0·50	0·60	0·50	0·45	0·51
8	0·65	0·75	—	—	—	—	—
9	2·15	1·95	2·05	2·00	2·35	2·00	2·10
10	0·80	0·60	0·95	1·10	0·70	0·75	0·88
11	0·30	0·05	0·30	0·10	0·25	0·15	0·20
12	0·80	0·70	0·70	0·40	0·65	1·05	0·70
Mean (all subjects)	0·84	0·71	0·84	0·77	0·85	0·80	—
Difference Sd̄ 0·0415 $P < 0·025$							
Mean for subjects 1–4 and 9–12	0·90	0·75	0·86	0·78	0·88	0·84	—

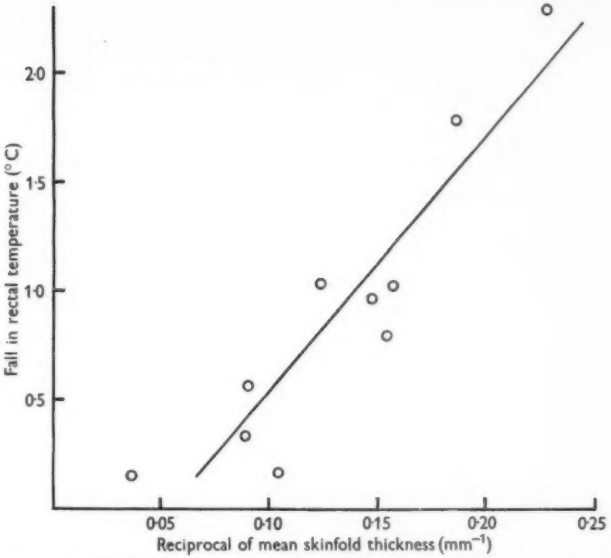


Fig. 1. The relationship between the men's subcutaneous fat thickness and their fall in rectal temperature during the 30 min immersions in stirred water at 15° C. ('Mean skinfold thickness' is the mean of sixteen readings at four standard sites. 'Fall in rectal temperature' is the mean fall in the man's last four immersions.)

TABLE 5. Rate of heat loss (cal/cm²/min) from the index finger 8 min after immersion

			Activity during morning before immersion				
Subject	Initially hot	Initially cold	Still, warm	Still, cold	Working, warm	Working, cold	Mean for individual
1	0·004	0·000	0·002	0·000	0·002	0·002	0·002
2	0·007	0·004	0·002	0·005	0·005	0·000	0·003
3	0·014	0·004	0·005	0·002	0·000	0·002	0·002
4	0·009	0·000	0·002	0·004	0·004	0·004	0·004
5	0·002	0·000	0·002	0·005	0·002	0·002	0·003
6	0·014	0·000	—	—	—	—	—
7	0·012	0·000	0·026	0·011	0·021	0·008	0·017
8	0·014	0·000	—	—	—	—	—
9	0·014	0·000	0·002	0·000	0·002	0·002	0·002
10	0·012	0·002	0·002	0·014	0·002	0·007	0·006
11	0·012	0·000	0·004	0·002	0·000	0·004	0·003
12	0·014	0·004	0·002	0·002	0·007	0·004	0·004
Mean	0·011	0·001	0·005	0·005	0·005	0·003	—
	Difference highly significant						
Mean for subjects 1–4 and 9–12	—	—	0·003	0·004	0·003	0·003	—

illustrated by the results from subjects 3 and 7, who had an almost identical skinfold thickness; subject 7 suffered the larger falls in temperature and had appreciably higher heat losses from his finger.

Tables 6 and 7 give the men's metabolic rates during immersion. These were considerably lower, both in the first 10 min and in the subsequent

TABLE 6. Metabolic rates (kcal/min) during 1st 10 min of immersion

			Activity during morning before immersion				Mean for individual	
Subject	Initially hot	Initially cool	Still, warm	Still, cold	Working, warm	Working, cold	kcal/min	kcal/m²/min
1	2·88	3·43	2·66	2·87	2·54	3·13	2·80	1·38
2	2·34	2·61	1·89	2·28	2·05	2·27	2·12	1·20
3	2·71	3·88	2·77	3·43	3·30	3·19	3·17	1·73
4	3·36	4·19	4·05	3·85	4·09	3·59	3·90	2·17
5	2·13	2·54	2·41	2·92	2·34	2·84	2·63	1·55
6	2·51	1·77	—	—	—	—	—	—
7	2·33	2·40	2·42	2·57	2·37	2·50	2·47	1·18
8	1·82	2·15	—	—	—	—	—	—
9	4·44	4·69	3·82	4·62	4·02	4·57	4·26	2·37
10	3·83	6·34	3·74	4·58	3·53	4·21	4·02	2·10
11	3·05	3·94	2·74	3·63	3·27	3·42	3·27	1·74
12	2·27	2·48	2·46	2·75	2·28	2·87	2·59	1·46
Mean (all subjects)	2·81	3·37	2·90	3·35	2·98	3·26	—	—
	Difference $\bar{Sd}$ 0·225 $P < 0·05$		Difference $\bar{Sd}$ 0·111 $P < 0·005$		Difference $\bar{Sd}$ 0·119 $P < 0·01$			
Mean for subjects 1–4 and 9–12	3·11	3·95	3·02	3·50	3·14	3·41	—	—
			Difference $\bar{Sd}$ 0·134 $P < 0·01$					

TABLE 7. Metabolic rates (kcal/min) between 10th and 30th min of immersion

			Activity during morning before immersion				Mean for individual	
Subject	Initially hot	Initially cool	Still, warm	Still, cold	Working, warm	Working, cold	(kcal/min)	(kcal/m²/min)
1	2·25	2·82	1·83	2·64	1·77	2·75	2·25	1·11
2	2·29	2·35	1·80	1·56	1·55	3·22	2·03	1·15
3	4·22	4·96	3·36	2·65	2·52	3·55	3·02	1·65
4	5·89	6·07	5·66	6·62	5·68	6·31	6·07	3·37
5	2·61	4·73	4·28	4·64	3·52	3·98	4·11	2·42
6	3·03	2·45	—	—	—	—	—	—
7	2·26	1·92	1·94	2·28	1·86	2·20	2·07	0·99
8	1·67	2·98	—	—	—	—	—	—
9	5·55	5·64	4·50	4·89	5·03	5·31	4·93	2·74
10	5·59	8·50	6·36	6·99	6·07	5·76	6·30	3·30
11	3·38	3·69	2·92	4·11	3·37	3·73	3·53	1·88
12	2·34	2·74	2·22	2·83	2·88	2·82	2·69	1·52
Mean (all subjects)	3·42	4·07	3·49	3·92	3·43	3·96	—	—
	Difference $\bar{Sd}$ 0·293 $P < 0·05$		Difference $\bar{Sd}$ 0·178 $P < 0·05$		Difference $\bar{Sd}$ 0·181 $P < 0·025$			
Mean for subjects 1–4 and 9–12	3·94	4·60	3·58	4·04	3·61	4·18	—	—
					Difference $\bar{Sd}$ 0·227 $P < 0·05$			

20 min of immersion, when the men were initially hot than when they were initially cool. They were also significantly lower after a morning in the warm than in the cold room, but work during the morning before immersion did not alter them significantly. These tables give the mean metabolic rate of each man during both the first 10 and the 20 min of his last four immersions and show that the thin men generally had higher metabolic rates in the water than the fat men. In the first 10 min the metabolic rate expressed as kcal/m²/min (surface area calculated from height and weight by the nomogram of Hawk, Oser & Summerson (1947)) depended comparatively little on the fat thickness of the individual (slope of line $b = 5{\cdot}26$ kcal/min/(fat thickness, mm)$^{-1}$, $P < 0{\cdot}05$), and there were large and fairly consistent individual differences in metabolic rate which were not related to fat thickness or to the fall in rectal temperature. For example, subject 11 had a higher metabolic rate than subject 12 in every experiment,

TABLE 8. Oxygen deficit of expired air, and change in heart rate and ventricular extrasystoles during immersion

			Activity during morning before immersion			
	Initially hot	Initially cool	Still, warm	Still, cold	Working, warm	Working, cold
Oxygen deficit of air expired during 1st 10 min of immersion						
Mean	2·68	3·06	2·85	2·98	2·84	3·02
Range	1·53–4·04	1·67–4·10	2·06–4·28	2·32–4·01	2·17–4·01	2·02–4·16
Difference SE 0·136 $P < 0{\cdot}025$						
Oxygen deficit of air expired between 10th and 30th minute of immersion						
Mean	4·07	4·04	4·02	3·91	3·89	4·09
Range	2·23–5·10	2·70–4·42	3·54–4·60	3·13–4·64	3·03–4·60	2·74–4·83
Change in heart rate during 1st minute of immersion						
Mean	+ 8·6	+ 16·6	+ 9·9	+ 7·5	+ 8·8	+ 11·0
Range	− 9·0 to + 19·2	− 5·4 to + 50·1	− 11·9 to + 29·8	− 19·5 to + 34·2	− 24·3 to + 34·9	− 17·3 to + 33·6
Ventricular extrasystoles 15–70 sec after immersion						
Total number	2	9	9	3	0	8
No. of men who developed them	1	5	3	1	0	3

both in absolute terms and in relation to surface area, in spite of his smaller falls in rectal temperature and greater fat thickness. In the last 20 min of immersion, although these individual differences persisted, the metabolic rates of the thin men rose while those of the three fattest men fell somewhat, and the men's metabolic rate was considerably more affected by their fat thickness (slope of line $b = 11{\cdot}84$ kcal/min/(fat thickness, mm)$^{-1}$, $P < 0{\cdot}025$). In the first 2–3 min of immersion only occasional irregular bursts of shivering were seen; the thinner men then started to shiver steadily and with progressively greater intensity as the experiment proceeded. All except those with the largest falls in temperature ceased to shiver immediately they left the tank.

Table 8 shows that the oxygen deficit of the expired air was significantly

lower during the first 10 min of immersion, though not in the next 20 min, when the men were initially hot than when they were initially cool. The men's change in heart rate was not significantly affected by their initial thermal state. Work and exercise on the morning before immersion had no significant effect on either the oxygen deficits or on the change in heart rates, and neither of these changed significantly with successive immersions. The table also gives the number of ventricular extrasystoles recorded between 15 and 70 sec after immersion; only those which could be identified with confidence as grossly abnormal ventricular complexes are recorded. Six men developed a total of 31 ventricular extrasystoles during this period, but their numbers are too few to draw conclusions about their frequency in different types of experiment. Extrasystoles were observed later in immersion and before immersion in only one man, subject 3; none were observed on this man between the 15th and 70th seconds of any immersion.

DISCUSSION

It is clear that the rate at which each man's rectal temperature fell during these immersions depended principally on the thickness of his subcutaneous fat. The fact that the relationship between their falls in temperature and the reciprocal of their fat thickness was approximately linear, small differences of fat thickness in thin men having a proportionately large effect on their fall in temperature, implies that skin and subcutaneous fat provided a major part of even the thin men's total tissue insulation. Presumably the insulation of their deeper tissues was low during these experiments as a result of a high muscle blood flow; Burton & Bazett (1936) have shown that shivering causes a decrease in total body insulation, presumably by this means. Extrapolation of the line relating fall in temperature to reciprocal fat thickness (see Fig. 1) suggests that an infinitely fat man would have increased his temperature by 0·62° C; this is almost the increase that would be expected in 30 min for a totally insulated man of 85 kg with a metabolic rate of 2·0 kcal/min, allowing for some respiratory heat loss. (In practice such a rise would presumably cause vasodilatation and so limit itself; this may have happened to some extent with subject 1, the fattest.) This, together with the fact that blood flow in the men's fingers virtually ceased within a few minutes of immersion, suggests that their skin and subcutaneous fat had a low blood flow and provided nearly their maximal insulation under the conditions of these experiments. However, peripheral blood flow was maintained somewhat better when the men were hot than when they were cool before immersion, and was probably responsible for their slightly more rapid falls in rectal temperature when they were hot; their lower metabolic rates when they were hot probably tended to reduce their falls in deep temperature

(Keatinge, 1959). Differences in peripheral blood flow also appeared to be responsible for some individual differences in the rate of fall of the rectal temperature, but they were much less important than subcutaneous fat thickness in this respect.

The large number of ventricular extrasystoles in the first 70 sec of the immersions confirms the previous observation (Keatinge, 1959) that they may occur on sudden immersion in cold water. They provide evidence of increased cardiac irritability, and the probable causes of this and its possible bearing on the problem of sudden death in cold water have been discussed already.

A fall in deep temperature, without cutaneous stimulation, can cause shivering (Sherrington, 1923–4), but observations that rats (Davies & Mayer, 1955), dogs (Good & Sellers, 1957*a*, *b*) and man (Uprus, Gaylor & Carmichael, 1935; Spurr, Hutt & Horvath, 1957) can be made to shiver by cutaneous cold when their deep body temperatures are unchanged or even raised have been regarded as evidence that the effect of deep temperature is of little importance in the metabolic response to cold. In the present experiments the importance of cutaneous stimulation was shown by the fact that men often ceased to shiver as soon as they left the water, in spite of moderate falls in rectal temperature. However, Carlson (1954) reported that men exposed to cold air shivered more readily when they had drunk cold water than when they had not, and the present experiments provide further evidence that the intensity of the metabolic response to cutaneous cold is greatly reduced by a small increase in the deep body temperature; cutaneous stimulation must have been greater, if anything, when the men were initially hot, but their metabolic rate was lower. Since the men's early response to cold was poorly related to their subcutaneous fat thickness and to their subsequent falls in rectal temperature, and there were large individual differences in metabolic rate during this time which were not related to either, their metabolic response to cutaneous cold clearly was poorly adjusted to their need to produce heat. As their deep temperatures fell the metabolic rates of the thin men increased and a continuing fall in deep temperature must ultimately have caused them to increase until each man achieved thermal balance, if balance was possible. The effect of a fall in deep body temperature would, then, presumably be the principal factor enabling the thin men to achieve thermal stability in a cold environment. These experiments, therefore, provide evidence that deep receptors play the main part in adjusting the metabolic rates of fat and thin men to their differing rates of heat loss during prolonged exposures to cold.

The men's metabolic rate during immersion was also substantially increased by exposure to cold earlier in the day. By the time of immersion

both their skin and rectal temperatures had returned to much the same level after 'cold' as after 'warm' mornings, but even the small and statistically insignificant difference in average temperatures that was still present might indicate a small but real difference in hypothalamic temperature and the possibility that a persistent change in hypothalamic temperature was responsible for the effect of a morning in the cold cannot be entirely excluded. The increased metabolic response in water after a morning in the cold room was not a consequence of the muscular work of shivering in the cold room, since a similar period of moderate exercise in the warm had no such effect.

This increased metabolic rate in the water produced by exposure to cold air earlier in the day contrasts with a diminution in metabolic response produced earlier by repeated brief immersions in water at the same temperature (Keatinge, 1959). Such opposite modification of the metabolic response by different types of exposure to cold is unlikely to take place in the cutaneous receptors. It may be related to the men's need to shiver or work in order to maintain their deep temperatures in the cold room, while shivering was certainly unnecessary and was probably, like work, undesirable during the brief immersions (Keatinge, 1959). The central nervous system would thus merely be modifying the metabolic response in the direction which was found to be appropriate in the previous exposure to cold. Brief repeated immersion of men in water at 15° C reduced their increase in heart rate and over-ventilation, as well as their metabolic rates, in the water; the heart rate and blood-pressure responses to immersion of a hand in either painfully hot or painfully cold water are also reduced by repetition (Glaser & Whittow, 1957; Glaser, Hall & Whittow, 1959), and such reductions may represent a general central nervous reaction to repeated brief unpleasant stimuli. In contrast, previous exposure to cold air in the present experiments increased the metabolic response to immersion without affecting other responses, probably through a conditioned reflex.

SUMMARY

1. The fall in the rectal temperature of each of ten young men immersed motionless for 30 min in stirred water at 15° C varied little in successive immersions and was closely related to the man's subcutaneous fat thickness by the equation $T = 11{\cdot}7/F - 0{\cdot}62$ (where T = fall in rectal temperature in °C and F = mean skinfold thickness in mm, measured at four standard sites).

2. The falls bore relatively little relation to the men's finger blood flow, which was always low during immersions, but both were slightly greater when the men were hot than when they were cool at the time of immersion.

3. The men's metabolic rates during immersion were substantially lowered by a small increase in their body temperatures at the time of immersion, and were increased by exposure to cold air, though not by moderate exercise, several hours before immersion.

4. In the first 10 min of immersion the metabolic rates of thin men were little higher than those of fat men, and there were a number of substantial and consistent individual differences in metabolic rate which were not related to the individual's fat thickness or to his fall in rectal temperature. In the last 20 min of immersion the metabolic rates of the thin men increased substantially but those of fat men did not.

I am indebted to Professor R. A. McCance for his support, to Dr M. Evans for assistance in the collection of the expired air, to Dr R. Lawn for operating the electrocardiograph, and to the subjects for their co-operation. I am also indebted to Surgeon-Captain F. E. Ellis for his interest and for making available the naval volunteers.

REFERENCES

ADOLPH, E. F. & MOLNAR, G. W. (1946). Exchanges of heat and tolerances to cold in men exposed to outdoor weather. *Amer. J. Physiol.* **146**, 507–537.

BROWN, G. M. & PAGE, J. (1952–3). The effect of chronic exposure to cold on temperature and blood flow of the hand. *J. appl. Physiol.* **5**, 221–227.

BURTON, A. C. (1941). An analysis of the problem of protection of the aviator against cold, and the testing of the insulating power of flying clothing. *Nat. Research Council, Canada, Report* No. C2033.

BURTON, A. C. & BAZETT, H. C. (1936). A study of the average temperature of the tissues, of the exchanges of heat and vasomotor responses in man by means of a bath calorimeter. *Amer. J. Physiol.* **117**, 36–54.

CARLSON, L. D. (1954). The adequate stimulus for shivering. *Proc. Soc. exp. Biol., N.Y.*, **85**, 303–305.

CARLSON, L. D., HSIEH, A. C. L., FULLINGTON, F. & ELSNER, R. W. (1958). Immersion in cold water and body tissue insulation. *J. Aviat. Med.* **29**, 145–152.

DAVIS, T. R. A. & MAYER, J. (1955). Nature of the physiological stimulus for shivering. *Amer. J. Physiol.* **181**, 669–674.

EDWARDS, D. A. W., HAMMOND, W. H., HEALEY, M. J. R., TANNER, J. M. & WHITEHOUSE, R. H. (1955). Design and accuracy of calipers for measuring subcutaneous tissue thickness, *Brit. J. Nutr.* **9**, 133–143.

GLASER, E. M., HALL, M. S. & WHITTOW, G. C. (1959). Habituation to heating and cooling of the same hand. *J. Physiol.* **146**, 152–164.

GLASER, E. M. & WHITTOW, G. C. (1957). Retention in a warm environment of adaptation to localized cooling. *J. Physiol.* **136**, 98–111.

GOOD, A. L. & SELLERS, A. F. (1957*a*). Temperature changes in the blood of the pulmonary artery and left atrium of dogs during exposure to extreme cold. *Amer. J. Physiol.* **188**, 447–450.

GOOD, A. L. & SELLERS, A. F. (1957*b*). Effects of carbon dioxide, ephedrine and ilidar on skin, blood and rectal temperatures of unanaesthetized dogs exposed to extreme cold. *Amer. J. Physiol.* **188**, 451–455.

HATFIELD, H. S. (1949). A heat-flow meter. *J. Physiol.* **111**, 10–11*P*.

HAWK, P. B., OSER, B. L. & SUMMERSON, W. H. (1947). *Practical Physiological Chemistry*, 12 ed. York, Pa, U.S.A.: Maple Press Co.

KEATINGE, W. R. (1959). *The effect of work, clothing and adaptation on the maintenance of the body temperature in water and on reflex responses to immersion.* Ph.D. Thesis, University of Cambridge.

KEATINGE, W. R. & EVANS, M. (1958). Modification of acute reflex responses to cold by brief training in cold climate. *Lancet*, **275**, 1038–1041.

LE BLANC, J. (1956). Evidence and meaning of acclimatization to cold in man. *J. appl. Physiol.* **9**, 395–398.

PUGH, L. G. C. & EDHOLM, O. G. (1955). The physiology of channel swimmers. *Lancet*, **269**, 761–768.

SHERRINGTON, C. S. (1923–24). Notes on temperature after spinal transection, with some observations on shivering. *J. Physiol.* **58**, 405–424.

SPURR, G. B., HUTT, B. K. & HORVATH, S. M. (1957). Shivering, oxygen consumption and body temperatures in acute exposure of men to two different cold environments. *J. appl. Physiol.* **11**, 58–64.

UPRUS, V., GAYLOR, G. B. & CARMICHAEL, E. A. (1935). Shivering: a clinical study with especial references to the afferent and efferent pathways. *Brain*, **58**, 220–232.

WEIR, J. B. DE V. (1949). New methods for calculating metabolic rate with special reference to protein metabolism. *J. Physiol.* **109**, 1–9.

WINSLOW, C.-E. A., HERRINGTON, L. P. & GAGGE, A. P. (1937). Physiological reactions of the human body to varying environmental temperatures. *Amer. J. Physiol.* **120**, 1–22.

J. Physiol. (1960), **153**, *pp.* 179–198
With 7 text-figures
Printed in Great Britain

THYROXINE BINDING BY SERUM PROTEINS

By W. E. BALFOUR and H. E. TUNNICLIFFE
From the Physiological Laboratory, University of Cambridge

(*Received* 4 *April* 1960)

When human serum containing ^{131}I-labelled thyroxine is subjected to electrophoresis on paper strips in barbiturate buffer, it has been found (Gordon, Gross, O'Connor & Pitt-Rivers, 1952) that, at physiological concentrations, thyroxine is bound in the main to a thyroxine-binding protein (TBP). This has an electrophoretic mobility which lies between those of the α_1- and α_2-globulins.

As the thyroxine concentration is raised above physiological levels, a steadily increasing proportion of the thyroxine is bound by albumin (Robbins & Rall, 1955; Dowling, Freinkel & Ingbar, 1956). Sera from other mammals show similar behaviour but Tata & Shellabarger (1959) find that in birds, for fowl and duck, TBP is absent and thyroxine binding occurs only in the albumin zone.

In paper-strip electrophoresis only small volumes (0·05–0·1 ml.) of serum can be applied to the paper. In consequence, accurate quantitative estimation of those protein constituents which are present in very low concentration is particularly difficult. Moreover, measurement of the distribution of radioactivity on the strip by scanning with a Geiger-Müller tube is also handicapped by the low sensitivity of the tube to γ-radiation. This low sensitivity limits the permissible fineness of the end-window slit and so hinders resolution. At low thyroxine concentrations competitive absorption of the thyroxine by the paper itself may cause further difficulties. It was therefore decided to investigate the binding of thyroxine by the technique of electrophoresis on cellulose columns (Porath, 1956; Gedin & Porath, 1957). These are capable of handling large volumes of serum (10–20 ml. or more) and any radioactivity associated with the eluted protein fractions can be determined in a well-type scintillation counter with high sensitivity to γ-radiation. We have also used a variety of buffers for the electrophoretic fractionation.

METHODS

Labelling of thyroid hormones with ^{131}I. Adult male rabbits (2–3 kg) were given an intraperitoneal injection of 0·25 mc of carrier-free ^{131}I as iodide; 48 hr later the animals were bled and the serum separated. In experiments with thyrotrophic hormone, the animal received the usual dose of ^{131}I; 48 hr later it was given a single intramuscular injection of 5 U.S.P.

units of thyrotrophic hormone (Armour) in 0·5 ml. of NaCl soln. 0·9 % (w/v). After a further 26 hr the serum was collected.

Isolation of labelled thyroid hormone from serum. Serum from the ^{131}I-treated animals was dialysed for 36 hr at 5° C against 2 l. borate-phosphate buffer, pH 8·6. 3 ml. of the dialysed serum was extracted 4 times with *n*-butanol: 0·3 mg thiouracil was added to the combined butanol extract (16 ml.) which was then evaporated to dryness at 45° C in a stream of N_2. The residue was taken up in 0·4 ml. of a mixture of ethanol (1 vol.) and 2 N-NH_4OH (4 vol.), and subjected to ascending chromatography on Whatman No. 1 paper, solvent system *n*-butanol (10 vol.), ethanol (2 vol.) and 2 N-NH_4OH (4 vol.). Marker spots of L-thyroxine and of the extract were located on the chromatogram with ninhydrin. The main body of the chromatogram was cut into narrow strips for serial determination of radioactivity in the scintillation counter. A single peak of radioactivity was detected: the active material in the relevant strips was extracted with *n*-butanol and rechromatographed, using as solvent system 2,4,6-collidine (100 vol.) and water (35 vol.) in an atmosphere of NH_3. Estimation of radioactivity in serial strips of this second chromatogram again showed a single peak corresponding in position with a marker spot of L-thyroxine.

Identification of L-thyroxine in pre-albumin fractions. Combined pre-albumin fractions from the electrophoresis of 10 ml. human serum containing 8 μg of added labelled thyroxine were concentrated to 2 ml. under reduced pressure at 40° C in a stream of N_2. The concentrate was extracted with *n*-butanol as described for rabbit serum; the butanol extracts, which contained 90 % of the radioactivity of the pre-albumin fractions, were concentrated and the majority of the concentrate applied as a single spot to Whatman No. 1 paper for ascending chromatography with solvent system *n*-butanol (10 vol.), ethanol (2 vol.) and 2 N-NH_4OH (4 vol.). The remainder of the butanol concentrate was mixed with authentic L-thyroxine and subjected to ascending chromatography in the same solvent system. Counting of serial strips of the first chromatogram revealed a single peak of radioactivity, which corresponded exactly with the position of two adjacent marker spots of L-thyroxine. In the second chromatogram the radioactivity (derived from the pre-albumin fractions) coincided exactly in position with the location of the ninhydrin reaction (due to the added carrier thyroxine). No other radioactivity was detected in either chromatogram.

Preparation of ^{131}I-labelled thyroxine. L-Thyroxine labelled with ^{131}I was prepared in early experiments by the method of Gross & Leblond (1950) and in later experiments by the method of Critchlow & Goldfinch (1954). The final product from either method was further purified by chromatography with the butanol solvent system. As required, labelled thyroxine was eluted from the appropriate area of the chromatogram with 2 ml. 2 N-NH_4OH, and the eluate was reduced in volume to a small drop by evaporation at 37° C at reduced pressure in a stream of N_2. When it was desired to increase the total thyroxine, the eluate of labelled thyroxine was supplemented with a portion of a solution of unlabelled thyroxine in 2 N-NH_4OH. The mixed sample was reduced in volume as described.

Electrophoresis. 10 ml. of serum (rabbit or pooled normal human) was added to the concentrated thyroxine preparation and allowed to stand, with gentle agitation, for 1 hr. The serum in a cellophane sac was then dialysed during the night against 2 l. of buffer. After dialysis the volume of the serum was measured, 0·5 ml. removed for measurement of total radioactivity, and the known residual volume put on to the cellulose column for electrophoresis. Cellulose was prepared by the method of Campbell & Stone (1956) and the columns (150 cm long × 2 cm diameter) were similar to those described by Gedin & Porath (1957). Electrophoresis continued for 40–48 hr, usually at 2000 V. On completion, elution with buffer solution commenced at a rate of 5 ml./10 min and eluted fractions were collected in an automatic fraction cutter.

Buffer solutions. (i) Borate-phosphate: 2·34 g $NaH_2PO_4 . 2H_2O$ + 4·29 g $Na_2B_4O_7 . 10\,H_2O$/l. (ii) Barbiturate: 1·23 g 5.5-diethyl barbituric acid + 6·87 g sodium 5.5-diethyl barbiturate/l. (iii) Phosphate: 1·56 g $NaH_2PO_4 . 2H_2O$ + 0·399 g NaOH/l.

Measurement of radioactivity. Samples of the eluted fractions were counted in a well-type scintillation counter; counts were corrected for background and decay.

Estimation of proteins. Two methods were used, (i) Weichselbaum's (1946) biuret method, and (ii) the method of Lowry, Rosebrough, Lewis-Farr & Randall (1951) with the Folin–Ciocalteu reagent. Method (ii) was employed where the protein content of the fractions was in the range 0–150 μg/ml., i.e. below the sensitivity of the biuret method. In all cases absorption at the appropriate wave-length was read on a Hilger spectrophotometer and the methods were calibrated against standard solutions of Armour's crystalline bovine serum albumin.

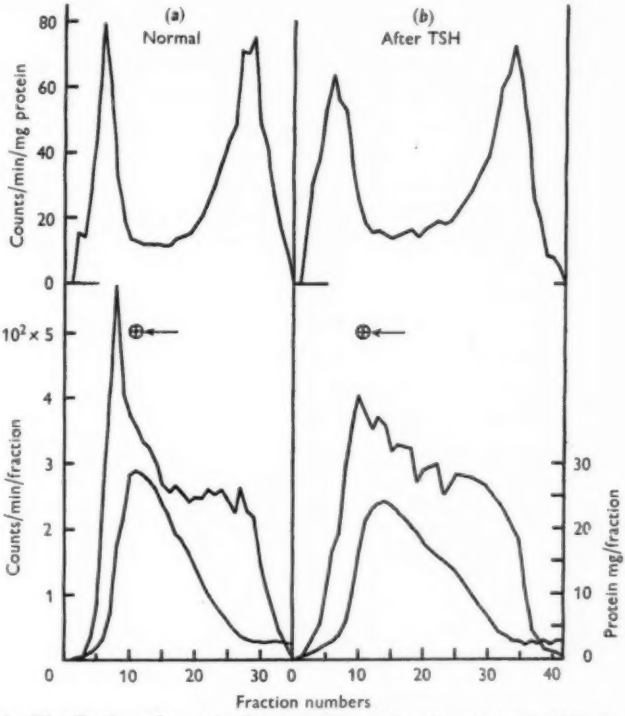


Fig. 1. Distribution of protein (lower curves) and radioactivity (middle curves) after zone electrophoresis of 10 ml. rabbit serum in borate-phosphate buffer pH 8·6. ⊕ ← indicates direction of electrophoretic migration. In (*a*) serum contained a normal amount of thyroxine which was labelled *in vivo* by a tracer dose of ^{131}I as iodide. In (*b*) serum was obtained as in (*a*) but after an injection of TSH. Upper curve in (*a*) and (*b*) is a plot of the ratios [radioactivity/fraction]: [protein/fraction]. Volume of each eluted fraction 3·5 ml. in (*a*), 4·0 ml. in (*b*).

RESULTS

Rabbit serum

Distribution of thyroxine in serum. Chromatography in two different solvent systems of the butanol extract of dialysed serum taken from a

rabbit after a tracer dose of ^{131}I showed that more than 90 % of the total radioactivity was due to thyroxine (see Methods). Since no other localization of radioactivity was detected, the distribution of radioactivity in the protein fractions obtained by electrophoresis of the serum indicates the distribution of the thyroid hormone, i.e. thyroxine, normally present in rabbit serum. Figure 1 (*a*) illustrates the thyroxine distribution in a normal rabbit which had received a tracer dose of ^{131}I and Figure 1 (*b*) the distribution when the labelled serum was taken from an animal which had also received a single dose of thyroid-stimulating hormone (TSH). Electrophoresis was carried out in borate-phosphate buffer pH 8·6. In both experiments the radioactivity/fraction rises sharply to a first peak which precedes the first (albumin) peak of the fractionated proteins. The radioactivity then falls sharply whilst the protein in the fractions is still rising. Thereafter the radioactivity declines more gradually until its final fairly steep fall to zero. The total radioactivity recovered in the fractions accounted in (*a*) for 93 % and in (*b*) for 92 % of the original radioactivity in the serum put on the column for electrophoresis. No radioactivity was found associated with fractions (not illustrated) which extended over the whole range of the β- and γ-globulins. For each fraction the value of the ratio [radioactivity/fraction]: [protein/fraction] was calculated, and the values obtained, which give a measure of the amount of thyroxine bound/mg protein, are shown in the upper curves of Fig. 1 (*a*) and (*b*). Three features are evident. There is first a pre-albumin zone where the thyroxine bound/mg protein reaches a peak in a protein fraction which has migrated ahead of the main bulk of the albumin. There follows an intermediate zone, extending over the crest of the albumin and for several fractions beyond, in which the amount of hormone bound/mg protein remains fairly constant at a considerably lower level.

Finally, the radioactivity/mg protein rises to a second maximum in the post-albumin region. In Fig. 1 (*a*), where the concentration of thyroxine is that normally present in rabbit serum, the maximum value for hormone bound/mg protein in the pre-albumin zone just exceeds the corresponding value in the post-albumin zone. Where the thyroid gland has been stimulated by TSH, the post-albumin maximum now exceeds that in the pre-albumin zone (Fig. 1*b*).

Figure 2 shows the results of fractionation of serum when the concentration of thyroxine was raised by the addition of labelled thyroxine 0·9 μg/ml. (Fig. 2*a*) and 1·9 μg/ml. (Fig. 2*b*). The features already described are again obvious but it is now evident that, in the post-albumin zone the radioactivity/fraction shows a moderate (Fig. 2*a*) or marked rise (Fig. 2*b*). This change is reflected in the values for the hormone bound/mg protein, which show that the post-albumin maximum value now definitely

exceeds the corresponding pre-albumin level, by 1·5 : 1 in Fig. 2(*a*) and by 4:1 in Fig. 2(*b*). Even at these elevated serum thyroxine concentrations we found no binding of thyroxine to the β- and γ-globulin fractions, and the total radioactivity recovered in the fractions illustrated accounted for 95·8 and 93 % of the thyroxine in the serum put on the column for electrophoresis.

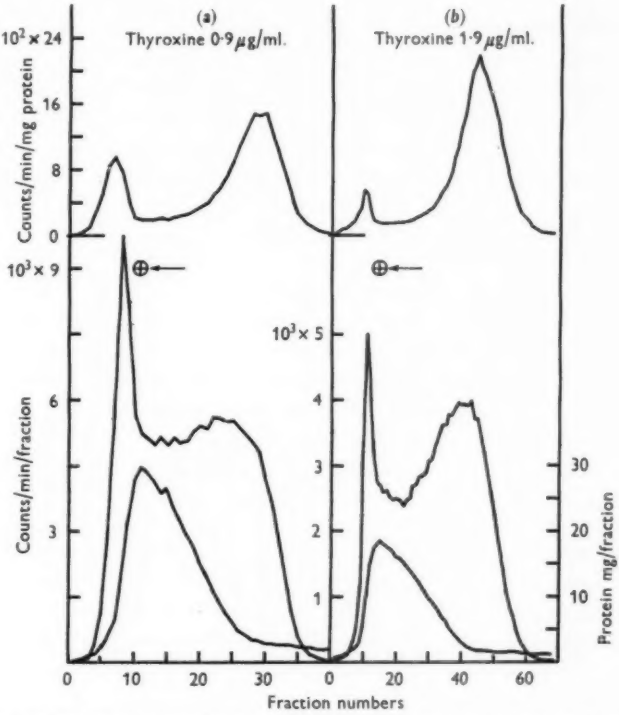


Fig. 2. Distribution of protein (lower curves) and radioactivity (middle curves) after zone electrophoresis of 10 ml. rabbit serum in borate-phosphate buffer pH 8·6. ⊕ ← indicates direction of electrophoretic migration. In (*a*) 0·9 μg thyroxine/ml. and in (*b*) 1·9 μg thyroxine/ml. were added to the serum. Upper curves in (*a*) and (*b*) are a plot of the ratios [radioactivity/fraction]: [protein/fraction]. Volume of each eluted fraction 3·5 ml. in (*a*), 2·5 ml. in (*b*).

In our hands the cellulose column with borate-phosphate buffer has not proved effective in separating the α_1- and α_2-globulins of rabbit serum and thus we cannot be certain that the rabbit post-albumin fraction which binds thyroxine has a mobility which is intermediate between those of the α_1- and α_2-globulins. But this post-albumin protein fraction certainly migrates

faster than the β-globulins and more slowly than the albumin, and seems to correspond with the thyroxine-binding protein of human serum. Myant & Osorio (1959), using paper strip electrophoresis in barbiturate buffer, report that in the adult rabbit thyroxine is bound by a serum protein (adult TBP) which moves between albumin and the α_1-globulin.

The relative totals of thyroxine bound in the pre- and post-albumin zones can be estimated approximately as follows. In Fig. 1(*a*) the total radioactivity attributable to the pre-albumin binding can be taken to be equal to the number of counts/min in peak fraction 8, together with twice the sum total of counts/min in fractions 1–7 inclusive. For the post-albumin binding the total can be taken to be the number of counts/min in peak fraction 27 together with twice the sum total of count/min in fractions 28–35 inclusive. The result indicates a relative binding of thyroxine pre-albumin: post-albumin of 1:1·3 when the hormone concentration is 0·05 μg/ml. serum, the normal endogenous level.

From Fig. 2(*b*) the pre-albumin binding is similarly given by the number of counts/min in peak fraction 11 together with twice the sum total of counts/min in fractions 1–10 inclusive and the post-albumin value by the number of counts/min in peak fraction 43 together with twice the sum total of counts/min in fractions 44–68 inclusive. In this case the relative binding of thyroxine pre-albumin: post-albumin is 1:5·5 at a hormone concentration raised to 1·95 μg/ml. serum.

In rabbit serum, then, we find clear evidence that thyroxine is bound in three zones: (i) by a pre-albumin protein which migrates electrophoretically very slightly faster than the main bulk of the albumin, (ii) by albumin itself and (iii) by a post-albumin protein. At normal endogenous concentrations of serum thyroxine the amount bound in the pre-albumin zone is $\sim \frac{3}{4}$ of that bound in the post-albumin zone: when the serum thyroxine concentration is greatly elevated a much greater proportion is bound in the post-albumin zone.

Human serum

Human serum was fractionated in borate-phosphate buffer pH 8·6 after the addition of labelled thyroxine, at total thyroxine concentrations of 0·1, 0·15, 0·2, 0·45, 0·85, 1·65, 2·45 and 8·05 μg/ml. serum. Figure 3 shows the distribution of radioactivity and of protein in the successive eluted fractions as far as the α_2-globulin fractions in four cases. The results shown are entirely typical of the remainder and are in all essentials similar to the results previously obtained (Balfour & Tunnicliffe, 1959) on a different sample of human serum. In each case there is a marked peak in the pre-albumin zone where the protein concentration is too small to be reliably estimated by the biuret method. Examination of these pre-albumin fractions by the far more sensitive Folin–Ciocalteu procedure shows that

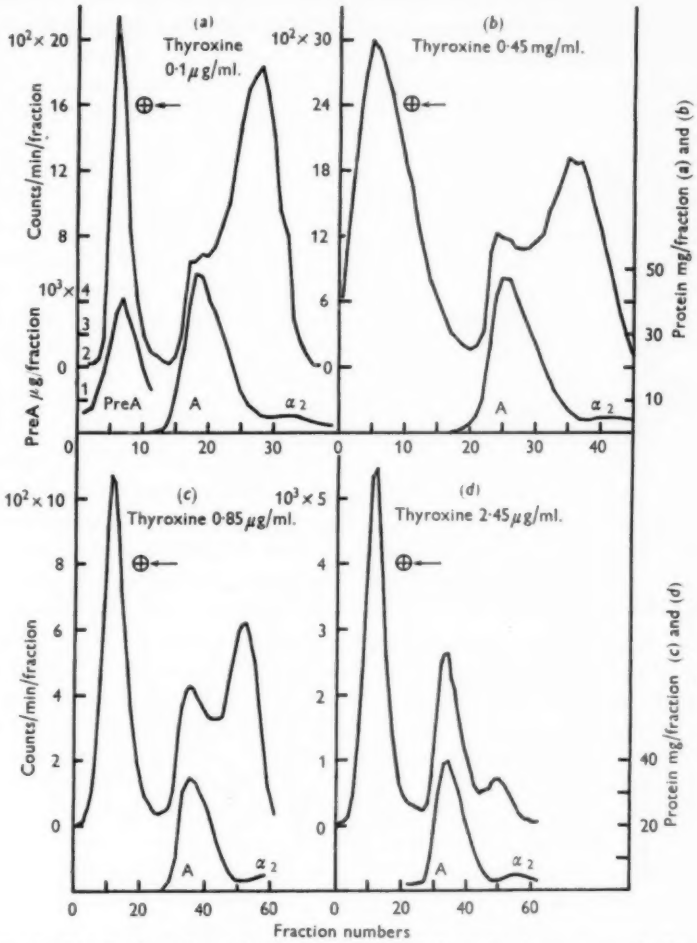


Fig. 3. Distribution of protein (lower curves) and thyroxine (upper curves) after zone electrophoresis of 10 ml. samples of human serum in borate-phosphate buffer pH 8·6. ⊕ ← indicates direction of electrophoretic migration. Concentrations of thyroxine were in (*a*) 0·1 μg/ml., (*b*) 0·45 μg/ml., (*c*) 0·85 μg/ml., and in (*d*) 2·45 μg/ml. PreA, A and α_2 indicate position of pre-albumin, albumin and alpha$_2$ globulin fractions. Note that pre-albumin concentration is expressed as μg/fraction: other proteins as mg/fraction. Volume of each eluted fraction 5·0 ml. throughout. Owing to variations in the specific activity of the thyroxine used it should be noted that the count rates/μg thyroxine differ widely in each of the experiments *a*, *b*, *c*, *d*.

there is a pre-albumin protein with its peak corresponding closely with the pre-albumin peak of radioactivity. Such a pre-albumin protein distribution is shown in Fig. 3(*a*).

The post-albumin zone shows a radioactive peak which at the lowest total level of thyroxine (0·1 μg/ml., Fig. 3*a*) is comparable in height with that due to the pre-albumin. As the total thyroxine/ml. serum is raised, the height of the post-albumin radioactivity peak, relative to the corresponding pre-albumin peak, diminishes progressively.

This post-albumin peak of radioactivity corresponds to a protein fraction which has electrophoretically migrated more slowly than the albumin fractions (from which the α_1-globulins have not fully separated) but faster than the α_2-globulin fractions. This post-albumin peak corresponds with the thyroxine-binding protein of other workers who have used paper electrophoretic fractionation.

Thyroxine binding by albumin is evident (Fig. 3*a*) as a distinct shoulder on the radioactivity curve in the region corresponding with the albumin protein peak. As the total thyroxine is raised, the binding of thyroxine by albumin is progressively more evident as a distinct peak (Fig. 3*b*, *c*) which, at a concentration of thyroxine of 2·45 μg/ml. (Fig. 3*d*), surpasses the relative TBP peak, though still smaller than the pre-albumin peak. At a total concentration of thyroxine of 8·05 μg/ml. the peak of radioactivity associated with the albumin becomes predominant and exceeds that due to the pre-albumin, while the post-albumin radioactivity is relatively reduced to a small shoulder on the descending limb of the albumin radioactivity curve. The better separation of the radioactivity into three zones in human serum, as compared with rabbit serum, makes easier the estimation of the partition of thyroxine between the pre-albumin, albumin and post-aibumin zones. The results are shown in Fig. 4. At a concentration of total thyroxine (0·1 μg/ml.) nearest to the natural endogenous level, the amount bound in the pre-albumin zone is about one half of that bound by the TBP. At 0·15 μg thyroxine/ml. the amounts bound by pre-albumin and TBP are equal and as the total thyroxine concentration is raised the amount bound by the pre-albumin continues to increase as far as the highest concentration (8·05 μg/ml.) examined. The rate of increase of pre-albumin-bound thyroxine is, however, falling off at the highest levels.

The amount of thyroxine bound by TBP, by contrast, reaches a fairly steady maximum level from a total concentration of 0·85 μg thyroxine/ml. onwards so that saturation of the binding capacity has clearly occurred.

The amount of thyroxine bound by albumin shows a steady increase with no indication of saturation over the range of thyroxine concentrations examined. The behaviour of human serum differs from that of rabbit serum when changes in binding at different thyroxine concentrations are com-

pared. In the rabbit the ratio pre-albumin bound thyroxine: post-albumin bound thyroxine changes from 1:1·3 to 1:5·5 as the total thyroxine concentration rises from ~ 0·05 to 1·95 μg/ml. In human serum the same ratio changes from 1:2 to 3·9:1 when the total thyroxine concentration rises from 0·1 to 1·65 μg/ml.

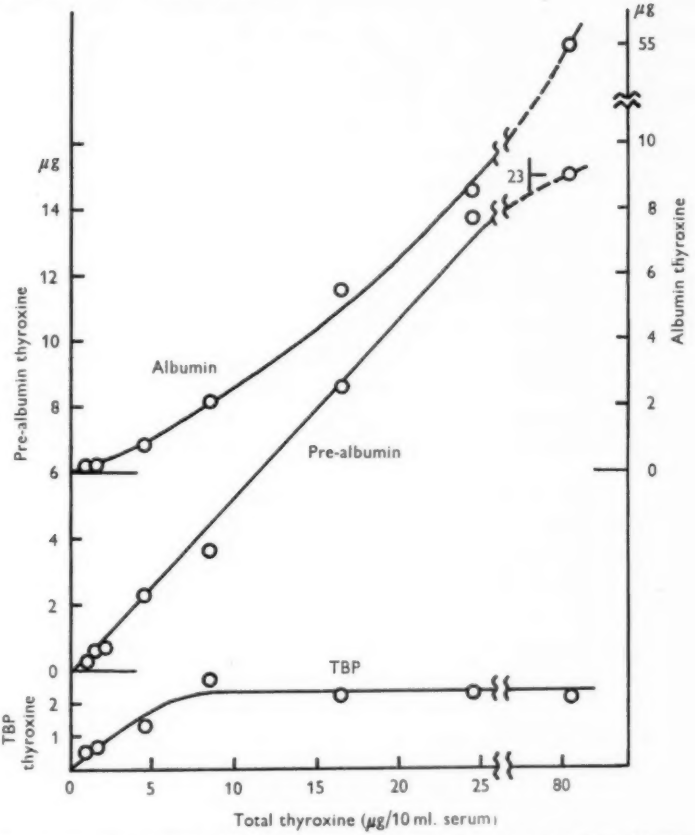


Fig. 4. Graphs showing the partition of thyroxine between the three binding proteins of human serum at different thyroxine concentrations. All values are expressed as μg thyroxine/10 ml. serum.

Thyroxine binding by pre-albumin

The magnitude of the pre-albumin bound radioactivity and the consistency with which we have been able to demonstrate it contrast strongly with the general failure of paper-strip electrophoresis to reveal it. Concentration of the pre-albumin fractions after column electrophoresis in borate-

phosphate buffer, followed by butanol extraction and chromatography, showed that thyroxine was the only source of the pre-albumin-bound radioactivity.

We have also measured the total pre-albumin present in the eluted pre-albumin fractions, and our results give, for the sample of mixed human serum used, a mean value of 0·26 mg pre-albumin/ml. serum. At the highest concentration of thyroxine used (8·05 μg/ml.) this pre-albumin binds 2·3 μg of thyroxine. Taking the mol. wt. of pre-albumin as 61,000, 1 mole is here binding 0·7 mole thyroxine. An estimate of the maximum binding capacity of pre-albumin was obtained in the following manner. Separated pre-albumin fractions (45 ml.) derived from 10 ml. of serum were collected. To 10 ml. of the combined fractions an excess (9·9 μg) of labelled thyroxine was added. After thorough mixing, 0·5 ml. was removed for counting, and the remainder (9·5 ml.) dialysed for 17 hr at room temperature against 500 ml. borate-phosphate buffer. Determination of radioactivity gave the results: pre-dialysis 5554 counts/min/0·5 ml.; post-dialysis 4373 counts/min/0·5 ml. Since 10 ml. of this serum contained 2·5 mg of pre-albumin, it may be calculated that 1 mole of pre-albumin is here binding 1·1 mole of thyroxine. At physiological levels of thyroxine concentration pre-albumin binding is of the order of 1 % of its capacity: i.e. only 0·01 mole thyroxine bound per mole of pre-albumin.

Thyroxine binding in the presence of other buffers

Phosphate. We have repeated the electrophoretic fractionation of human serum containing added labelled thyroxine in phosphate buffer solutions at pH 7·4 and 8·0. In both cases it was easy to demonstrate thyroxine binding in a pre-albumin zone, and the results of a typical experiment at pH 8·0 are shown in Fig. 5. The resolution of the albumin from the α-globulin protein fractions is poor in phosphate buffer, and as a result the radioactivity due to albumin and TBP binding of thyroxine shows as a single peak displaced to the right relatively to the main protein peak. The recovery of radioactivity in the eluted fractions was satisfactory (96–99 %), and at the concentration of thyroxine used (0·8 μg/ml. serum) the pre-albumin bound between 42 and 48 % of the total thyroxine. This result agrees well with the binding by pre-albumin in borate-phosphate buffer at a comparable thyroxine concentration.

Barbiturate. Two samples of the same human serum with the same concentration (1·65 μg/ml.) of added labelled thyroxine were subjected to electrophoresis (*a*) in borate-phosphate buffer pH 8·6 and (*b*) in barbiturate buffer pH 8·6, ionic strength 0·03. The results of the electrophoretic fractionation are shown in Fig. 6. In each case the protein fractionation shows a well marked pre-albumin peak, the usual large albumin zone and

a small α_2-globulin zone. The sharpness of the fractionation in the albumin and α-globulin region is better in the barbiturate buffer.

The distribution of radioactivity in the borate-phosphate experiment

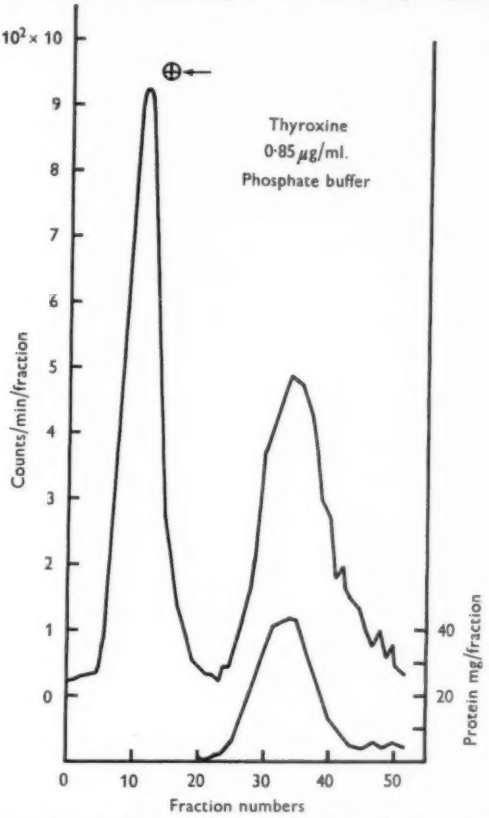


Fig. 5. Distribution of protein (lower curve) and thyroxine (upper curve) after zone electrophoresis of 10 ml. human serum in phosphate buffer pH 8·0. Serum contained 0·85 μg thyroxine/ml. ⊕ ← indicates direction of electrophoretic migration. Volume of each eluted fraction 5·0 ml.

(Fig. 6*a*) shows typically a strongly marked peak due to pre-albumin binding of thyroxine, a well marked peak due to albumin binding and a smaller peak due to TBP. In barbiturate buffer the results are strikingly different. For here, Fig. 6*b*, the radioactivity corresponding to the pre-albumin is reduced to an extremely low though just detectable level. The peak of radioactivity associated with the albumin is greatly enhanced and there is little apparent change in the TBP radioactivity.

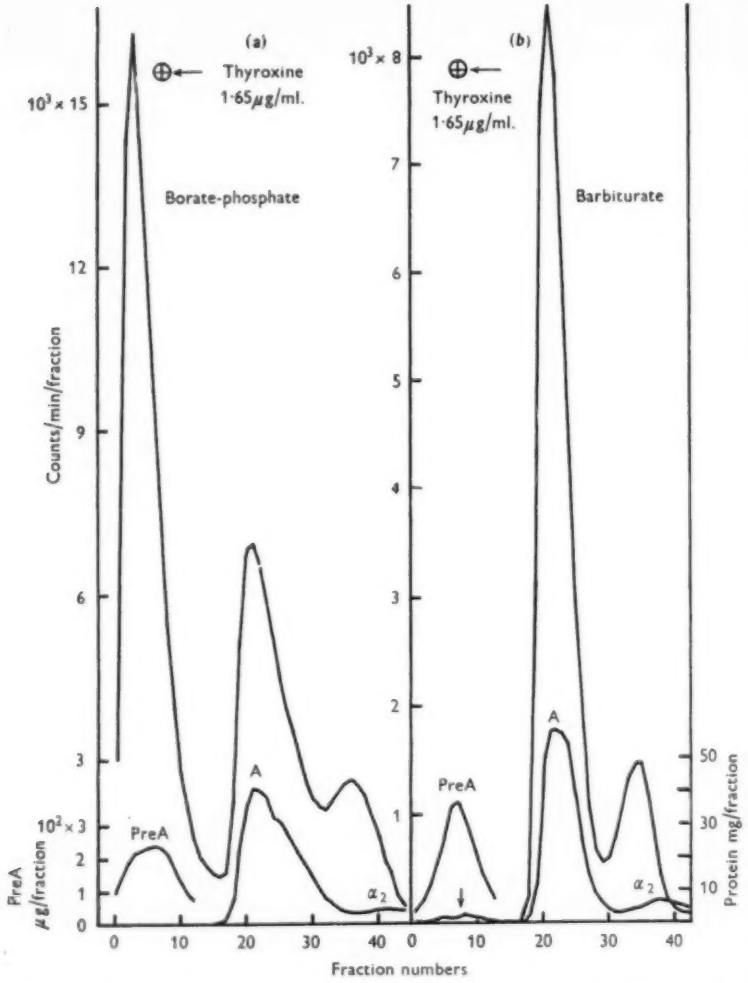


Fig. 6. Distribution of protein (lower curves) and thyroxine (upper curves) after zone electrophoresis at pH 8·6 of 10 ml. samples of human serum. Concentration of thyroxine 1·65 μg/ml. ⊕ ← indicates direction of electrophoretic migration. In (*a*) borate-phosphate buffer was used; in (*b*) barbiturate buffer. PreA, A and α_2 indicate position of pre-albumin, albumin and alpha$_2$ globulin fractions. Note that pre-albumin concentration is expressed as μg/fraction: other proteins as mg/fraction. Volume of each eluted fraction 5·0 ml. throughout. In (*b*) ↓ indicates small binding of thyroxine by pre-albumin.

Analysis of the distribution of thyroxine in the two experiments (Table 1) emphasizes the effect of barbiturate buffer. The barbiturate buffer almost completely prevents the binding of thyroxine by pre-albumin. It does not prevent thyroxine binding by TBP, which remains saturated, binding 0·23 μg thyroxine/ml. serum and 0·25 μg/ml. serum in the two cases, as would be expected from the results shown in Fig. 4 for the concentration of thyroxine (1·65 μg/ml. serum) used.

The effect of barbiturate buffer on the binding of thyroxine by pre-albumin was shown to be a direct effect, and not due indirectly to an enhancement of the binding power of albumin by the method to be described. Pre-albumin fractions were collected after serum electrophoresis in barbiturate buffer. One 10 ml. sample was dialysed during the night against 1 l. of borate-phosphate buffer in order to remove the majority of

TABLE 1. Distribution of thyroxine 1·65 μg/ml. in serum protein fractions following electrophoresis in borate-phosphate and barbiturate buffers pH 8·6

	Percentage of thyroxine carried by		
	Pre-albumin	Albumin	TBP
Borate-phosphate buffer	52·5	33·7	13·8
Barbiturate buffer	0·9	83·9	15·2

the barbiturate. To this dialysed sample, and to a second 10 ml. sample still in barbiturate buffer, ^{131}I-labelled thyroxine was added and each sample was dialysed during the night against 500 ml. of its own buffer solution. Determination of radioactivity before and after dialysis showed that in borate-phosphate buffer the isolated pre-albumin showed its normal binding capacity. In the presence of barbiturate buffer, thyroxine binding by the separated pre-albumin was depressed.

Duck serum

Duck serum with added labelled thyroxine was subjected to column electrophoresis in both borate-phosphate and barbiturate buffers. The results are shown in Fig. 7. It can be seen (Fig. 7*b*) that in barbiturate buffer there is one very prominent peak of radioactivity corresponding with the albumin fraction of the proteins. There is no sign of any radioactivity peak corresponding to TBP (i.e. in the post-albumin region) but there is to be seen a small hump on the pre-albumin region of the radioactivity curve corresponding to a small plateau in the pre-albumin region of the protein distribution curve.

When the results in borate-phosphate buffer are examined, using a sample of the same serum with the same concentration of added thyroxine, two main peaks of radioactivity are seen (Fig. 7*a*). The larger of the two lies

in the pre-albumin region and corresponds in position with the pre-albumin zone of the protein fractionation. The second, smaller peak corresponds to the albumin fraction of the proteins but is much reduced in size compared with the albumin radioactivity peak shown in barbiturate buffer. No TBP radioactivity peak is visible.

In borate-phosphate buffer duck serum thus binds thyroxine predominantly by pre-albumin, and to a lesser extent by albumin. In barbiturate

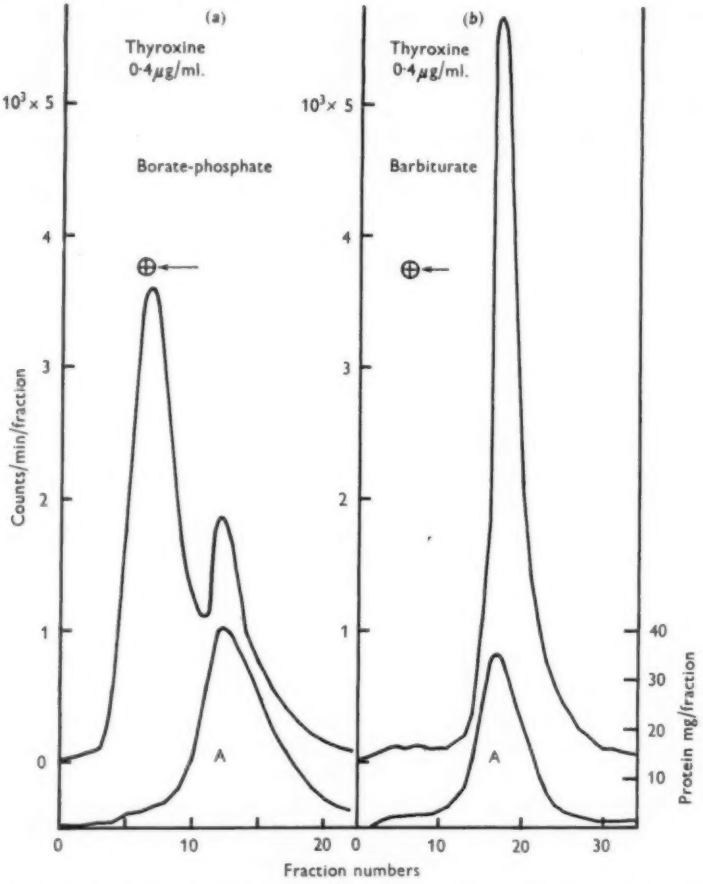


Fig. 7. Distribution of protein (lower curves) and thyroxine (upper curves) after zone electrophoresis at pH 8·6 of 10 ml. samples of duck serum. Serum contained 0·4 μg thyroxine/ml. ⊕ ← indicates direction of electrophoretic migration. In (*a*) borate-phosphate buffer was used: in (*b*) barbiturate buffer. A indicates albumin zone of serum proteins. Volume of each eluted fraction 5·0 ml. throughout.

buffer the pre-albumin binding is almost completely prevented (as in human serum), and owing to the absence of TBP the whole of the binding is now due to albumin.

DISCUSSION

Deutsch & Goodloe (1945) used free electrophoresis in a barbiturate–citrate system at pH 8·6 and demonstrated the presence of a fast-moving pre-albumin component in the sera of many animal species, particularly in birds. Hoch & Chanutin (1953) showed by free electrophoresis in 0·6 % NaCl solution at pH 8–8·3 that a pre-albumin component was present in human serum and plasma. They found a concentration range of 7–33 mg/100 ml. serum, with an average of ~ 20 mg/100 ml. Gedin & Porath (1957) detected a pre-albumin fraction in human serum by column electrophoresis; Ingbar (1958) demonstrated pre-albumin in human serum by column electrophoresis in tris-maleate buffer, and Halliday & Kekwick (1957) by free electrophoresis in phosphate buffer at pH 8·0 showed that sera of young rats contain a fast-moving component which migrates ahead of the main albumin peak.

Our own results with phosphate buffer at pH 7·4 and 8·0 and with barbiturate buffer pH 8·6 agree with these earlier results and the essentially identical results we have obtained by electrophoresis in a borate-phosphate buffer system make it clear that the presence in this system of a fast-moving pre-albumin component is not an artifact due to the choice of buffer. Nor is thyroxine binding by pre-albumin in borate-phosphate buffer an anomalous result. For, as we have shown, similar binding occurs in phosphate buffer: it occurs also in tris-maleate buffer (Ingbar, 1958) and as Beierwaltes & Robbins (1959) have very recently found, in an ammonium carbonate buffer system.

In contrast, paper-strip electrophoresis of serum generally fails to reveal the presence of a pre-albumin protein fraction (Robbins & Rall, 1955; Morris & Courtice, 1955; Tata, 1959*a*). Morris & Courtice did, however, observe that a pre-albumin fraction was constantly present in mouse plasma, and occasionally demonstrable in rat and guinea-pig plasma. It seems clear, however, that the conventional method of staining the paper strip with dyes is usually inadequate to detect pre-albumin separated from the small initial volume of serum applied to the paper.

But since pre-albumin has such a marked binding capacity for thyroxine it might, nevertheless, be expected that the pre-albumin zone on the paper strip would be detected by location of the associated radioactivity. TBP, which at many levels of thyroxine concentration is binding less thyroxine than pre-albumin, is so located. But there is general failure to find definite localization of radioactivity associated with a pre-albumin

zone on the paper strip. This failure arises, we suggest, from the conventional choice of barbiturate buffer for the electrophoretic fractionation.

We have shown that at an ionic strength of 0·03, barbiturate buffer effectively prevents pre-albumin binding of thyroxine. At the higher ionic strengths (0·05–0·1) usually used in paper-strip electrophoresis, the binding power of pre-albumin may well be sufficiently depressed to make the residuum of radioactivity associated with the pre-albumin usually not detectable by scanning with a Geiger–Müller tube. With scintillation counting after zone electrophoresis in a barbiturate buffer this small residual radioactivity is just recognizable, and there are occasional reports of weak pre-albumin-associated radioactivity on paper strips when barbiturate buffer has been used (Robbins, Rall & Petermann, 1957; Robbins & Nelson, 1958).

The effect of barbiturate buffer on pre-albumin binding has further important consequences when the *distribution* of thyroxine binding by the protein fractions of serum is studied. For in a barbiturate system that thyroxine which would have been bound by pre-albumin now becomes redistributed. At low thyroxine concentrations the redistributed thyroxine will be bound by both albumin and TBP. At high thyroxine concentrations, where the binding capacity of TBP is already saturated, the redistributed thyroxine becomes bound by albumin only. In short, a variably distorted picture of thyroxine binding by serum proteins results from electrophoretic fractionation in a barbiturate buffer system.

It is not surprising, therefore, that, in a borate-phosphate system, the distribution of thyroxine binding at different thyroxine concentrations differs markedly from that reported by Robbins & Rall (1955) and by Dowling *et al.* (1956), who used a barbiturate system. Since this precludes the considerable pre-albumin binding of thyroxine their findings are of limited validity. In particular, the significance of the changes in distribution of thyroxine binding in different physiological conditions reported by Robbins & Nelson (1958) and by Dowling *et al.* (1956) may need reassessment.

The contrast between thyroxine binding by albumin, TBP and pre-albumin in the borate-phosphate system is notable. The amount of thyroxine bound by albumin continues to increase up to the highest concentration (8·05 μg/ml. serum) of thyroxine examined. There is no indication of approaching saturation: this is to be expected from the results of Lein (1952) on thyroxine binding by bovine serum albumin. Lein's measurements were, however, made at the highly alkaline pH of 9·75 and may thus have caused opening of binding sites not available at a lower pH (Tata, 1959*b*). TBP becomes saturated when the thyroxine concentration has reached 0·85 μg/ml. and then carries 0·23 μg thyroxine/ml. Pre-albumin is still not saturated at 8·05 μg thyroxine/ml. serum but is,

nevertheless, then carrying more than ten times the amount of thyroxine carried by TBP.

Our results in borate-phosphate and phosphate buffers agree with those of Ingbar (1958), who found that, during column or paper-strip electrophoresis in tris-maleate buffer pH 8·6, labelled thyroxine migrated in association with serum proteins in the interalpha globulin zone and also in a pre-albumin zone. Ingbar points out that this observation is contrary to results in barbiturate buffer where the labelled thyroxine migrates principally in the interalpha zone (TBP). The blocking effect of barbiturate buffer which we have described explains the difference observed by Ingbar. Ingbar also found that, at very low concentrations of added thyroxine (0·4 μg/100 ml.) the percentage of labelled thyroxine associated with pre-albumin is as great or nearly as great as that associated with TBP. Again our results in borate-phosphate and phosphate buffers agree essentially with Ingbar's results in the tris-maleate system, and our value for the thyroxine-binding capacity of TBP agrees excellently with Ingbar's value, both results being $\sim$ 25 μg thyroxine/100 ml. serum. Our figure ($\sim$ 230 μg thyroxine/100 ml. serum) for the binding capacity of pre-albumin is, however, greater than the approximate value (100 μg thyroxine/100 ml. serum) given by Ingbar. This difference may, however, be due simply to the different samples of serum having different concentrations of pre-albumin; the results of Hoch & Chanutin (1953) suggest that a fourfold variation in different samples may well be possible.

The method for estimating pre-albumin was standardized against bovine serum albumin. In consequence our results for the quantity of pre-albumin present in serum rest on the assumption that the amount of chromogenic material per unit weight of protein is the same in bovine serum albumin and in pre-albumin. The calculation of the molar binding capacity of pre-albumin for thyroxine depends also on the assumption that the pre-albumin present in the eluted fractions from the column electrophoresis consists of a single molecular species with a molecular weight of 61,000. This is the value for the molecular weight of the pre-albumin isolated from human serum by Schultze, Schöneberger & Schwick (1956). Because of these assumptions, and of the evidence considered later in this discussion that there may be three pre-albumins in serum, we would stress that our value for the molar binding capacity of pre-albumin for thyroxine must be regarded as tentative.

Our results confirm the finding of Tata & Shellabarger (1959) that duck serum lacks TBP. Their statement that duck serum binds thyroxine by albumin only, though correct when barbiturate buffer is employed, is not correct when the serum is examined in borate-phosphate buffer. At a concentration of 0·4 μg thyroxine/ml. duck serum shows pre-albumin

binding of thyroxine to an extent considerably greater than that due to albumin. Tata & Shellabarger (1959) discuss the reasons for the relatively longer biological half-life of thyroxine, compared with triiodothyronine, in the mammal although in birds the biological half-life of the two compounds is the same. Their explanation of the difference is based on the absence of TBP and, as they presume, the sole binding of thyroxine and triiodothyronine by albumin in chick and duck sera. But their argument does not take into account the marked binding of thyroxine by pre-albumin (at any rate in the duck) and may therefore be not necessarily valid.

Schultze *et al.* (1956) isolated a human serum pre-albumin and Tata (1959*a*, *b*) found that this pre-albumin possessed strong thyroxine-binding power. Moreover, Tata gave good reasons for suggesting that a large part of this pre-albumin is present as a complex in the α-globulin fraction and that it is responsible for the binding of thyroxine observed in TBP.

Further work is needed to determine the relation of Schultze's pre-albumin to the pre-albumin fraction examined by us. For the present it may be pointed out that our pre-albumin fraction co-exists with TBP, and has its binding power greatly diminished in the presence of barbiturate buffer. This buffer does not prevent binding of thyroxine by TBP (i.e. by the complexed form of Schultze's pre-albumin): nor does barbiturate prevent thyroxine binding by the isolated form of Schultze's pre-albumin (Tata, 1959*a*). Moreover, as Ingbar (1958) has found, pre-albumin possesses essentially no binding power for triiodothyronine, whereas TBP binds it both in barbiturate and in tris-maleate buffer systems. The relation of both pre-albumins to the two pre-albumins observed in starch-gel electrophoresis of human serum by Poulik & Smithies (1958) and to the three pre-albumin zones described more recently by Poulik (1959) requires further examination. One of the two pre-albumin zones observed in starch-gel electrophoresis by Rich & Bearn (1958) was shown by them to bind thyroxine.

SUMMARY

1. Electrophoresis of rabbit, human and duck sera on cellulose columns with borate-phosphate buffer reveals the presence of a pre-albumin protein fraction (TBP) which binds thyroxine.

2. The pre-albumin fraction of human serum, and binding of thyroxine by this pre-albumin, have also been demonstrated by column electrophoresis in phosphate buffer.

3. Pre-albumin in rabbit serum and in human serum binds thyroxine to an important extent at physiological levels of serum thyroxine concentration.

4. The partition of thyroxine between pre-albumin, albumin and TBP

has been measured in human serum over a wide range of serum thyroxine concentration.

5. The thyroxine-binding capacity of the pre-albumin actually present in human serum greatly exceeds the binding capacity of the TBP also present in the same serum.

6. In the rabbit, when the thyroxine concentration in serum is increased, a much greater proportion of the thyroxine becomes bound by TBP relatively to that bound by pre-albumin. This behaviour is the reverse of that shown by pre-albumin and TBP in human serum.

7. Thyroxine binding by human and duck serum pre-albumin is prevented by the presence of barbiturate buffer solution.

8. Thyroxine binding by human TBP, and by human and duck serum albumin, is not prevented by the presence of barbiturate buffer solution.

9. Duck serum does not contain TBP.

10. Thyroxine binding in duck serum is, at a thyroxine concentration of 0·4 μg/ml., due predominantly to pre-albumin and to a lesser extent to albumin.

We are grateful to Dr G. S. Adair for suggesting modifications in the manuscript, and to Dr R. D. Keynes for the loan of a scintillation counter. Our thanks are also due to Mr A. J. Secker for technical assistance in the design and construction of the power unit used for electrophoresis.

REFERENCES

Balfour, W. E. & Tunnicliffe, H. E. (1959). Thyroxine binding by serum proteins. *J. Physiol.* **148**, 64–65*P*.

Beierwaltes, W. H. & Robbins, J. (1959). Familial increase in the thyroxine binding sites in serum alpha globulin. *J. clin. Invest.* **38**, 1683–1687.

Campbell, P. N. & Stone, N. E. (1956). Zone electrophoresis of plasma proteins. *Biochem. J.* **62**, 9*P*.

Critchlow, A. & Goldfinch, M. K. (1954). Preparation of thyroxine labelled with ^{131}I and its use as an analytical tool. In *Radioisotope Conference*, **1**, ed. Johnstone, J. E. London: Butterworth.

Deutsch, H. F. & Goodloe, M. B. (1945). An electrophoretic survey of various animal plasmas. *J. biol. Chem.* **161**, 1–20.

Dowling, J. T., Freinkel, N. & Ingbar, S. H. (1956). Thyroxine binding by sera of pregnant women, newborn infants and women with spontaneous abortion. *J. clin. Invest.* **35**, 1263–1276.

Gedin, H. I. & Porath, J. (1957). Zone electrophoresis in vertical columns. II. Zone electrophoresis of serum proteins. *Biochim. biophys. acta*, **26**, 159–169.

Gordon, A. H., Gross, J., O'Connor, D. & Pitt-Rivers, R. (1952). Nature of the circulating thyroid hormone–plasma protein complex. *Nature, Lond.*, **169**, 19–20.

Gross, J. & Leblond, C. P. (1950). Metabolism of thyroid hormone in the rat as shown by physiological doses of thyroxine. *J. biol. Chem.* **184**, 489–500.

Halliday, R. & Kekwick, R. A. (1957). Electrophoretic analysis of the sera of young rats. *Proc. Roy. Soc.* B, **146**, 431–437.

Hoch, H. & Chanutin, A. (1953). Electrophoretic studies on fast moving components of human serum. *J. biol. Chem.* **200**, 241–249.

Ingbar, S. H. (1958). Pre-albumin a thyroxine-binding protein of human plasma. *Endocrinology*, **63**, 256–259.

Lein, A. (1952). Thyroxine binding by bovine serum albumin. *Fed. Proc.* **11**, 91.

LOWRY, O. H., ROSEBROUGH, N. J., LEWIS-FARR, A. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–273.

MORRIS, B. & COURTICE, F. C. (1955). The protein and lipid composition of the plasma of different animal species determined by zone electrophoresis and chemical analysis. *Quart. J. exp. Physiol.* **40**, 127–137.

MYANT, N. B. & OSORIO, C. (1959). Serum proteins, including thyroxine-binding proteins, in maternal and foetal rabbits. *J. Physiol.* **146**, 344–357.

PORATH, J. (1956). Methodological studies of zone electrophoresis in vertical columns. I. Fractionation in cellulose powder columns of substances of low molecular weight exemplified by amino acids and related compounds. *Biochim. biophys. acta*, **22**, 151–175.

POULIK, M. D. (1959). Separation of pre-albumins by starch gel electrophoresis. *Nature, Lond.*, **184**, 1067–1068.

POULIK, M. D. & SMITHIES, O. (1958). Comparison and combination of the starch-gel and filter-paper electrophoretic methods applied to human sera: two-dimensional electrophoresis. *Biochem. J.* **68**, 636–643.

RICH, C. & BEARN, A. G. (1958). Localisation of the thyroxine-binding protein of serum by starch-gel electrophoresis. *Endocrinology*, **62**, 687–689.

ROBBINS, J. & NELSON, J. H. (1958). Thyroxine-binding by serum protein in pregnancy and in the new born. *J. clin. Invest.* **37**, 153–159.

ROBBINS, J. & RALL, J. E. (1955). Thyroxine-binding capacity of serum in normal man. *J. clin. Invest.* **34**, 1324–1330.

ROBBINS, J., RALL, J. E. & PETERMANN, M. L. (1957). Thyroxine-binding by serum and urine protein in nephrosis. Qualitative aspects. *J. clin. Invest.* **36**, 1333–1342.

SCHULTZE, H. E., SCHÖNENBERGER, M. & SCHWICK, G. (1956). Über ein Präalbumin des menschlichen Serums. *Biochem. Z.* **328**, 267–284.

TATA, J. R. (1959*a*). Pre-albumin as a complex in the α-globulin fraction in human serum. *Nature, Lond.*, **183**, 877–879.

TATA, J. R. (1955*b*). A new aspect of the interaction between thyroxine and proteins. *Biochem. J.* **72**, 222–229.

TATA, J. R. & SHELLABARGER, C. J. (1959). An explanation for the difference between the responses of mammals and birds to thyroxine and tri-iodothyronine. *Biochem. J.* **72**, 608–613.

WEICHSELBAUM, T. E. (1946). Determination of proteins in blood serum and plasma. *Amer. J. clin. Path.* **16**, 40.

J. Physiol. (1960), **153**, *pp.* 199–208
With 7 text-figures
Printed in Great Britain

FRUCTOSE METABOLISM IN THE ISOLATED PERFUSED LIVER OF THE FOETAL AND NEW-BORN SHEEP

By W. H. H. ANDREWS, H. G. BRITTON, A. ST G. HUGGETT AND D. A. NIXON

From the Departments of Pharmacology and Physiology, St Mary's Hospital Medical School, London, W. 2

(*Received* 21 *April* 1960)

While fructose is known to be a major component of the foetal blood in all species of Ungulata and Cetacea which have been examined, the mechanism by which it is formed is uncertain. Huggett, Warren & Warren (1951), Alexander, Andrews, Huggett, Nixon & Widdas (1955) and Alexander, Huggett, Nixon & Widdas (1955) showed that glucose was probably a precursor in its formation and that the site of synthesis was centred in the placenta. It was postulated that intermediate phosphate esters would participate in the synthesis of the free fructose. However, Hers (1957*a*) obtained evidence that fructose was formed from glucose in the seminal vesicle without the intervention of phosphorylated intermediates. In this pathway glucose is reduced to sorbitol by the enzyme aldose reductase, and the sorbitol is subsequently oxidized to fructose by the enzyme ketose reductase. Evidence was also obtained that aldose reductase, but not ketose reductase, was present in the sheep placenta. Since foetal liver slices were able to convert sorbitol to fructose, it was postulated that the fructose in the sheep foetus was formed in two stages: in the first stage sorbitol was formed from glucose in the placenta, and in the second stage sorbitol was converted to fructose in the foetal liver. This mechanism is shown schematically in Fig. 1, DPN and TPN referring to di- and tri-phosphopyridine nucleotides respectively.

The work of Alexander, Andrews *et al.* (1955) did not exclude the possibility that some foetal fructose is formed by the mechanism indicated above, and it was thought desirable to confirm the second stage of the Hers hypothesis on the perfused foetal liver. At the same time the uptake of fructose by this preparation was examined. This latter aspect was investigated since preliminary experiments had suggested that the foetal liver was unable to utilize fructose, in contrast to the adult liver where conversion to glucose may take place (Cori, Ochoa, Slein & Cori, 1951).

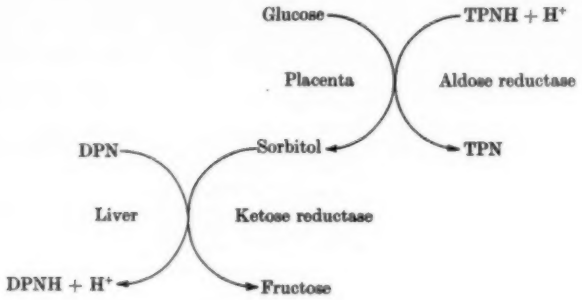


Fig. 1.

METHODS

Apparatus. The apparatus used to perfuse the livers of lambs was similar to that described by Andrews, Hecker & Maegraith (1956). The portal vein was supplied from a small, constant-level reservoir, the portal pressure was about 8 cm saline. The perfusing fluid was blood obtained from the lamb, supplemented with maternal blood. Thermometers placed between lobes of the liver registered 35·5–36° C.

Foetal livers were perfused with the apparatus illustrated in Fig. 2. Blood from the liver flowed to the reservoir from where it was pumped to a bubble oxygenator (moist gas, 5 % CO_2 + 95 % O_2), and allowed to overflow into a collection chamber which was coated with Antifoam A (Midland Silicones, Ltd.). The blood passed into a constant-level device which also acted as a bubble trap, and thence, via a heating coil, to the liver. A manometer was incorporated near the liver and the inflow pressure was controlled to 8 cm saline by a screw clip. The overflow from the constant-level device was returned to the reservoir. The foetus and reservoir were placed in a lagged bath of saline at 37° C. The apparatus was primed with heparinized maternal blood.

Operative technique. Lambs were given pentobarbitone (20 mg/kg body wt.) intraperitoneally as a basal anaesthetic and then anaesthetized with intravenous thiobarbitone. The trachea was cannulated and artificial respiration begun. A mid-line incision was made, avoiding the umbilicus, the sternum was divided in the mid line, and the ribs retracted laterally. The thrombosed umbilical vein was ligated and the rumen divided between ligatures. The portal vein was cleaned for about 1·5 cm of its length immediately cephalad to the pancreas. A cannula was inserted into the thoracic part of the posterior vena cava, pointing towards the liver, and blood from the posterior part of the body was allowed to flow into the reservoir. Immediately afterwards the portal vein was cannulated and the perfusion begun. Tributaries to the portal vein between the cannula and the porta hepatis were ligated. When the level of blood in the reservoir became constant the vena cava was clamped just posterior to the liver. The edges of the incision were drawn together. The total time for operation was about 25 min.

With the foetus, the head was submerged in saline, and 2 ml. of 2 % procaine was injected parenterally to prevent pain. A mid-line incision was made as in the procedure for lambs; the umbilical vein and thoracic posterior vena cava were cannulated; a clamp was applied to the vena cava posterior to the liver and the perfusion was started. Ligature of the portal vein appeared to be unnecessary. The time taken for the operation was approximately 6 min.

In both the lamb and foetal preparations a certain amount of mottling of the surface of the liver occurred, especially where the ribs appeared to be exerting slight pressure. Some

dilatation of the vessels took place during the first hour of the perfusion, since the rate of flow increased when the pressure was kept constant—most of this increase occurred in the first 20 min. Therefore 30 min was allowed to elapse between the beginning of the perfusion and the commencement of metabolic experiments. An injection of Indian ink, made at the end of the experiment, demonstrated that the inflowing blood rapidly penetrated to all areas of the liver in both preparations.

The volume of the perfusate was between 250 and 500 ml. and the rate of flow varied from 100 to 280 ml./min in different preparations. In the foetal liver preparations the weight of the organ ranged from 51 to 80 g, and in the neonatal livers from 150 to 190 g.

Chemical determinations. Fructose was determined by the method of Chinard, Danesino, Hartmann, Huggett, Paul & Reynolds (1956); glucose by the method of Huggett & Nixon (1957); lactic acid by the method of Barker & Britton (1957), except that the concentration of semicarbazide in the buffer solution was 0·5 g/100 ml.; and inositol by the microbiological method of Campling & Nixon (1954).

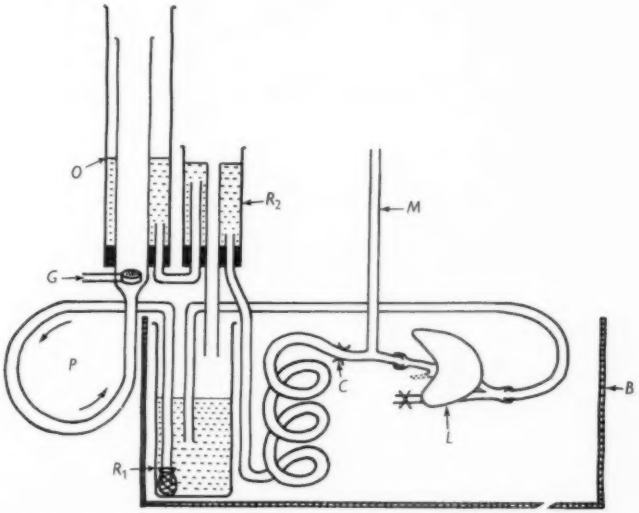


Fig. 2. A schematic diagram of the foetal or neonatal liver perfusion circuit. *B*, saline bath; *C*, screw clip; *G*, 95% O_2+5% CO_2; *L*, liver; *M*, manometer; *O*, oxygenator; *P*, pump; R_1, reservoir; R_2, constant-level reservoir and bubble trap.

RESULTS

Observations on perfused foetal livers

In the five foetal livers perfused, ranging from 122–140 days of foetal age, the ability of the liver to convert sorbitol to fructose was consistently demonstrated. Neither the addition of glucose nor of meso-inositol significantly influenced the level of the circulating fructose in the preparation, whereas the introduction into the circuit of an equivalent quantity of sorbitol resulted in a prompt production of fructose (Fig. 3*a*). The

magnitude of this conversion was in several cases such as to suggest a complete conversion of the added sorbitol to fructose. In one experiment the addition of galactose was also without effect upon the fructose concentration.

The blood used to prime the circuit was obtained from an adult ewe and therefore the foetal liver was subjected to a perfusate having a concentration of fructose lower than that encountered in sheep foetal blood. It might therefore be suggested that the sorbitol–fructose conversion only proceeded under conditions of a low concentration of blood fructose, and that the reaction was a mechanism ensuring the characteristic high blood fructose concentration encountered in the sheep foetus. However, the

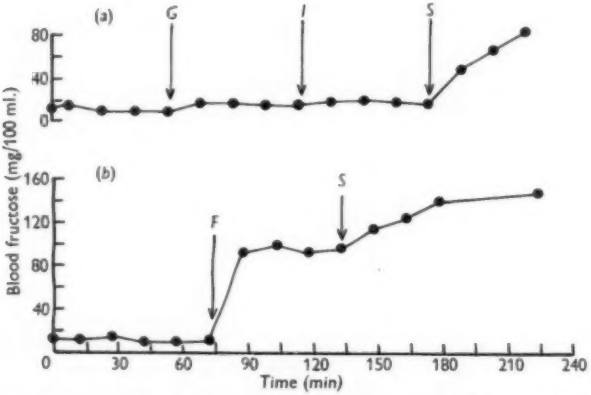


Fig. 3. The elevation of the fructose concentration in the perfusate of the foetal sheep liver preparation following the addition of sorbitol. (*a*) Concentration of fructose in the perfusate following the addition of 500 mg of glucose, *G*, inositol, *I*, and sorbitol, *S*. Foetal age 122 days. Liver weight 61 g. Terminal perfusate volume 410 ml. (*b*) Concentration of fructose in the perfusate following the addition of sorbitol (*S*, 500 mg) subsequent to the elevation of the fructose concentration by the addition of 500 mg of fructose, *F*. Foetal age 140 days. Liver weight 80 g. Terminal perfusate volume 500 ml.

ability of the liver to convert sorbitol to fructose still took place when the concentration of fructose was elevated to a level comparable to that found in the normal foetus (Fig. 3*b*).

The initial rate of conversion of sorbitol to fructose ranged from 4·2 to 13·3 mg/min, and was unrelated to foetal age. The glucose concentration in the perfusing blood showed an increase with time. The initial concentrations ranged from 84 to 232 mg/100 ml. (mean 116) and during the course of the experiments the maximum increase observed was 124 mg/100 ml.

In two experiments the concentration of lactic acid in the perfusing blood was measured. In both cases the values showed a fall in concentration 60–80 min after the start of the perfusion, with restoration of the initial value after a further 60 min. In one experiment the initial value was 72, the lowest value 32 and the final value 99 mg/100 ml. at 205 min. In the other experiment the initial value was 25·2 mg/100 ml. and the final value, after 200 min, was 28·5 mg/100 ml. (illustrated in Fig. 4).

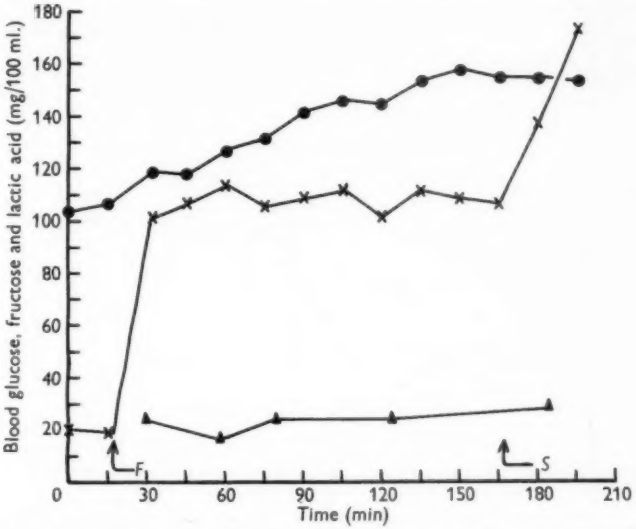


Fig. 4. Concentrations of glucose, fructose and lactic acid in the perfusate of the foetal liver after the addition of 500 mg fructose, *F*, and 500 mg sorbitol, *S*. Foetal age 134 days. Liver weight 51 g. Terminal perfusate volume 460 ml. ×, Fructose; ●, glucose; ▲, lactic acid.

It will be seen from Fig. 3*b* that the elevated fructose concentration remained constant over the 60 min before the introduction of sorbitol. The inability of the perfused foetal sheep liver to utilize fructose under these experimental conditions was confirmed in two further preparations, where the elevated fructose concentration was observed over a longer period of time, one of which is shown in Fig. 4. In the other preparation the concentration fluctuated between 90 and 94 mg/100 ml. over 120 min.

An increase in the inositol concentration of the perfusing blood was observed in the three experiments where it was determined. The rate of production over the first hour varied from 5·0 to 7·5 mg inositol/hr. This apparent synthesis of inositol by the liver is possibly an artifact, since it is to be remembered that the perfusion circuit was primed with maternal

blood, considerably lower in inositol concentration than foetal blood (Campling & Nixon, 1954); thus the observed rise may merely represent the elution of inositol from a tissue initially in equilibrium with a higher inositol concentration. When inositol was added to the perfusate the concentration subsequently showed no significant change (Fig. 5).

The additions of sorbitol, glucose or fructose were without significant effect upon the inositol concentrations present in the perfusing blood.

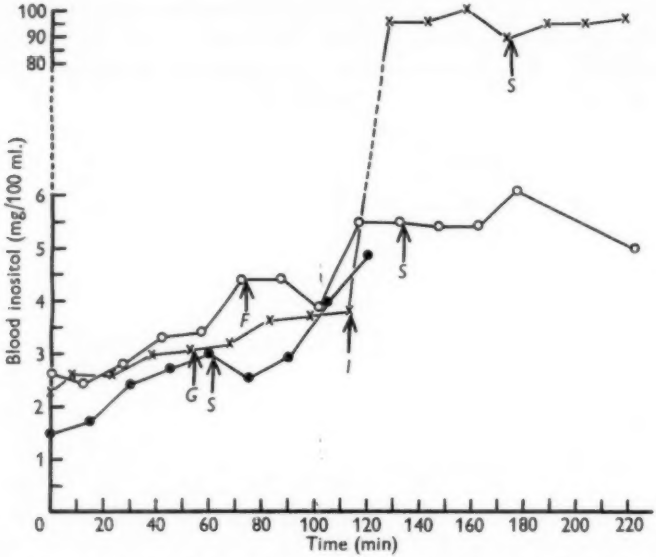


Fig. 5. Concentration of meso-inositol in the perfusate of foetal livers. At *G*, *S*, *F* and *I* 500 mg of glucose, sorbitol, fructose or meso-inositol respectively were introduced to the perfusate. ×, Foetal age 122 days. Liver wt. 61 g. Terminal perfusate vol. 410 ml. ●, Foetal age 138 days. Liver wt. 75 g. Terminal perfusate vol. 400 ml. ○, Foetal age 140 days. Liver wt. 80 g. Terminal perfusate vol. 500 ml.

Observations on perfused lamb livers

The inability of the preparation to utilize fructose to a substantial degree was still present in a lamb 3 days after birth. By the fifth day, however, fructose utilization appeared to be well established (Fig. 6). A similar prompt decline in the elevated blood fructose was also observed in livers from 9- and 15-day-old lambs.

Lactic acid concentrations in the perfusate were found to be lower than those encountered in foetal preparations. A substantially higher concentration of blood glucose was present in all the lamb perfusions,

compared with the foetal liver preparations. The response of the liver to sorbitol was again the production of fructose, although the concentration rise was smaller due to its metabolism.

In one experiment on the intact anaesthetized lamb, 10 days after birth, fructose was rapidly removed from the circulation following its experimental elevation. Renal loss of fructose over the initial 74 min following the administration amounted to 17·4% of the injected dose. Ligation of the renal pedicles did not greatly influence the decline of blood fructose concentration, suggesting that the main loss of the

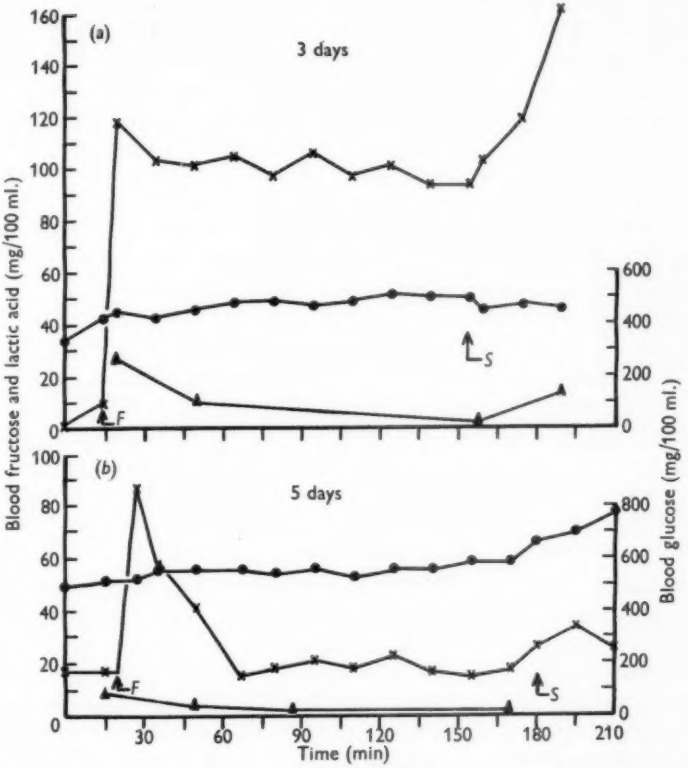


Fig. 6. Fructose and glucose and lactic acid concentrations in the perfusate following the addition of 500 mg fructose, *F*, and 500 mg sorbitol, *S*, to neonatal preparations. (*a*) Response 3 days after birth. Liver weight 150 g. Terminal perfusate volume 380 ml. (*b*) Response 5 days after birth. (The apparent high control values for fructose are due to interference by the high concentration of glucose and other chromatogenic substances in the perfusate.) Liver weight 165 g. Terminal perfusate volume 350 ml. ×, Fructose; ●, glucose; ▲, lactic acid.

administered fructose was extra-renal. An elevation of the fructose concentration again occurred following the injection of sorbitol. The elevation of the glucose concentration following ligature of the renal pedicles was probably a reflexion of the trauma induced by the procedure.

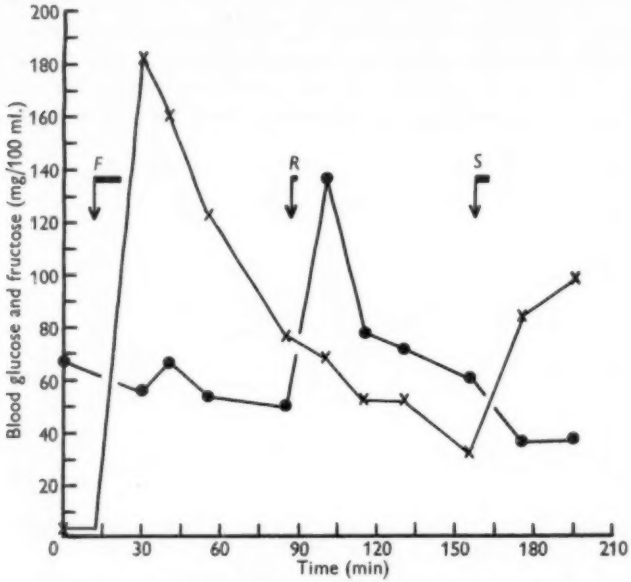


Fig. 7. Fructose and glucose concentrations in the blood of an anaesthetized 10-day new-born lamb following the intravenous injection of 3·8 g fructose, *F*, and 3·8 g sorbitol, *S*, and ligation of the renal pedicles, *R*. ×, Fructose; ●, glucose.

DISCUSSION

Embden & Griesbach (1914) demonstrated a production of fructose from sorbitol in the perfused dog liver. The results presented in this paper, obtained by using a perfused foetal sheep liver preparation, indicate that this conversion of sorbitol to fructose can occur in foetal life and thus confirm the observations of Hers (1957*a*) who used foetal sheep liver slices. Although the conversion has been shown to be possible it, of course, does not imply that the fructose present in sheep foetal blood is formed by the liver from sorbitol. Studies on the perfused sheep placenta show that little sorbitol is formed compared to the amount of fructose produced, at least in the initial period of the perfusion (Andrews, Britton & Nixon, 1959). Thus in a typical experiment the fructose concentration in the perfusion circuit rose by 54 mg/100 ml. in the first hour of perfusion while

the sorbitol concentration increased by 10 mg/100 ml. This suggests that the amount of fructose formed by the liver from sorbitol, in the intact animal, is small.

Blakley (1951) showed that the conversion of sorbitol to fructose in the adult was brought about by the enzyme ketose reductase (sorbitol dehydrogenase). A similar enzyme has been demonstrated in the foetal sheep liver from 90 days onward by H. G. Britton (unpublished), using the method of Neil, Walker & Warren (personal communication).

The inability of the foetal liver to take up fructose contrasts strongly with the neonatal liver a few days after birth. It is believed that the first step in the metabolism of fructose is the phosphorylation of the sugar to fructose-1-phosphate by adenosine triphosphate in the presence of fructokinase (Hers, 1955), and it is perhaps relevant that Hers (1957*b*) was unable to demonstrate fructokinase activity in the foetal sheep liver. Whether the uptake of fructose by the lamb liver is paralleled by the appearance of this enzymic activity in the tissue is, however, not known.

The constancy of the lactic acid concentrations suggests that foetal livers were able to remove lactic acid produced by glycolysis occurring in the blood. Since the blood lactic acid concentrations in the lamb liver perfusions were lower than in the foetal liver preparations the ability of the foetal liver to take up lactic acid may be limited. If this is the case it may provide an explanation for the fact that lactic acid concentration in the foetal blood usually exceeds that of the mother (Barker & Britton, 1958), and also for the maintenance of high lactic acid concentration induced by hypoxia (Britton, Nixon & Wright, 1959).

From the perfused liver experiments, where no hormone additives are present, there would appear to be a developmental pattern in the establishment of hepatic metabolism. Thus, by the 122nd day of foetal life the liver is capable of converting sorbitol to fructose, yet the ability of the tissue to remove fructose to any substantial degree does not appear to be present until after birth.

SUMMARY

1. Perfusion circuits suitable for metabolic investigations have been developed for the foetal or neonatal sheep liver.

2. It has been shown that sorbitol can be converted to fructose by the preparation. Glucose, galactose and inositol did not increase the fructose concentration in the perfusate.

3. Under these experimental conditions the foetal liver appears to be unable to metabolize fructose, but rapid utilization cannot be detected in the perfused neonatal liver before the fifth day after birth.

We wish to acknowledge the support of this work by a Medical Research Council grant and the technical assistance of J. R. Hancock and Miss Margaret Trent.

REFERENCES

ALEXANDER, D. P., ANDREWS, R. D., HUGGETT, A. St G., NIXON, D. A. & WIDDAS, W. F. (1955). The placental transfer of sugars in the sheep: studies with radioactive sugar. *J. Physiol.* **129**, 352–366.

ALEXANDER, D. P., HUGGETT, A. St G., NIXON, D. A. & WIDDAS, W. F. (1955). The placental transfer of sugars in the sheep: the influence of concentration gradient upon the rates of hexose formation as shown in umbilical perfusion of the placenta. *J. Physiol.* **129**, 367–383.

ANDREWS, W. H. H., BRITTON, H. G. & NIXON, D. A. (1959). The role of sorbitol in the formation of foetal fructose in the sheep. *J. Physiol.* **146**, 41–42*P*.

ANDREWS, W. H. H., HECKER, R. & MAEGRAITH, B. G. (1956). The action of adrenaline, noradrenaline, acetylcholine and histamine on the perfused liver of the monkey, cat and rabbit. *J. Physiol.* **132**, 509–521.

BARKER, J. N. & BRITTON, H. G. (1957). The enzymatic estimation of L (+)-lactic acid. *J. Physiol.* **138**, 3–4*P*.

BARKER, J. N. & BRITTON, H. G. (1958). Lactate and pyruvate metabolism in the foetal sheep. *J. Physiol.* **143**, 50–51*P*.

BLAKLEY, R. L. (1951). The metabolism and antiketogenic effects of sorbitol. Sorbitol dehydrogenase. *Biochem. J.* **49**, 257–271.

BRITTON, H. G., NIXON, D. A. & WRIGHT, G. H. (1959). Hypoxic death in the foetal sheep. *J. Physiol.* **149**, 37–38*P*.

CAMPLING, J. D. & NIXON. D. A. (1954). The inositol content of foetal blood and foetal fluids. *J. Physiol.* **126**, 71–80.

CHINARD, F. P., DANESINO, V., HARTMANN, W. L., HUGGETT, A. St G., PAUL, W. & REYNOLDS, S. R. M. (1956). The transmission of hexoses across the placenta in the human and the rhesus monkey (*Macaca mulatta*). *J. Physiol.* **132**, 289–303.

CORI, G. T., OCHOA, S., SLEIN, M. W. & CORI, C. F. (1951). The metabolism of fructose in liver. Isolation of fructose-1-phosphate and inorganic pyrophosphate. *Biochim. biophys. acta*, **7**, 304–317.

EMBDEN, G. & GRIESBACH, W. (1914). Über Milchsaure und Zuckerbildung in der isolierten Leber. I. Über den Abbau der D. Sorbose. II. Über das Schicksal des D. Sorbit und einiger anderer Hexite. *Hoppe-Seyl. Z.* **91**, 251–286.

HERS, H. G. (1955). *Methods in Enzymology*, vol. 1, ed COLOWICK, S. P. and KAPLAN, N. O. New York: Academic Press Inc.

HERS, H. G. (1957*a*). *Le metabolisme du fructose*. Bruxelles: Editions Arscia.

HERS, H. G. (1957*b*). Presence of sorbitol in seminal vesicles and in foetal blood of the sheep. *Biochem. J.* **66**, 30*P*.

HUGGETT, A. St G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase and *o*-dianisidine in determination of blood and urinary glucose. *Lancet*, **273**, 368–370.

HUGGETT, A. St G., WARREN, F. L. & WARREN, N. V. (1951). The origin of the blood fructose of the foetal sheep. *J. Physiol.* **113**, 258–275.